Extraction, Isolation and Biological studies of *Pentanisia prunelloides* and *Hippobromus pauciflorus*

University of Fort Hare
Together in Excellence

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By

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To God.

To my ancestors.

To my family.

To my supervisor and co-supervisor for their help.

To my friends and laboratory mates.

To everyone who helped ease the burden of this work.

Abstract

Enormous amounts of drugs or drug components have been found with the use and help of ethnobotanical knowledge of plants by the indigenous civilisation of that region. The primary reason that plants are chosen as a potential source of new medicines is that they are readily available to researcher and the public either free of charge or at a low and affordable cost in the rural majority community in the developing world.

The aim of this study was to evaluate the chemical content of two commonly used medicinal plants of the Eastern Cape Province of South Africa and to explore their antioxidant potential.

Crude extracts of *Hippobromus pauciflorus* and *Pentanisia prunelloides* were obtained by means of sequential solvent extraction followed by evaluation of the phenolic and flavonoid content of extracts and the anti-oxidant ability of the both plant samples and also their ability to hinder lipid peroxidation.

For the *H.pauciflorus* sample, different masses of crude extracts were obtained with Dichloromethane (0.817 g) yielding the smallest mass and the 70% Ethanol (120.196 g) extract yielding the largest mass.

The total phenolic content of the plant sample extracts were measured using the Folin-Ciocalteau reagent. The Methanol extract (133.019 ± 0.003 μgGAE/mL) exhibited the largest content while the EtOH extract (13.981 ± 0.001 μgGAE/mL) exhibited the least content.

The MeOH extract exhibited the highest flavonoid content (46.005 ± 0.001 μgQE/mg extract) while the Hexane extract (19.000 ± 0.008 μgQE/mg extract) exhibited the least..

Frap assay was performed with the Ethyl Acetate extract displaying the least anti-oxidant ability (10.284 ± 0.014 AAE/mL) while the Hex extract (204.705 ± 0.119 AAE/mL) displayed the highest anti-oxidant ability.

2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed with the hexane, methanol and ethanol extracts exhibiting an anti-oxidant property with IC_{50} values of 3.594x10-3 ± 0.133 mg/mL, 0.185 ± 0.019 mg/mL and 0.011 ± 0.002 mg/mL respectively.

Inhibition of lipid peroxidation at 150 μg/mL was performed where the Dichloromethane extract showed the highest inhibition percentage (-75.000 ± 0.119%) while the Hex extract (-41.667 ± 0.021005%) showed the lowest inhibition percentage at 300 μg/mL; only the DCM extract (-3.051 ± 0.018%) displayed inhibition ability.

.For the *P.prunelloides* sample, different masses of crude extracts were obtained with EA extract (0.348 g) yielding the smallest mass and the MeOH (47.941 g) extract yielding the largest mass.

Phenolic content was evaluated with the DCM extract (122.827 ± 0.010 μgGAE/mL) exhibited the largest content while the MeOH extract (48.788 ± 0.001 μgGAE/mL) exhibited the least content.

Flavonoid content was evaluated with the DCM extract (88.543 ± 0.005 μgQE/mg extract) exhibited the largest content while the EtOH extract (19.254 ± 0.001 μgQE/mg extract) exhibited the least content.

FRAP assay was performed with the DCM extract (13.021 ± 0.008 AAE/mL) displaying the least anti-oxidant ability while the MeOH extract (217.758 ± 0.025 AAE/mL) displaying the highest anti-oxidant ability.

DPPH assay was conducted with EA and methanol extracts exhibiting anti-anti-oxidant ability and having IC_{50} values of 0.579 ± 0.021 mg/mL and 0.006± 0.001 mg/mL respectively.

Inhibition of lipid peroxidation at 150 μg/mL was performed with the n-Hex extract (-36.395 ± 0.015%) showing the highest inhibition percentage while the DCM extract (-17.647 ± 0.005%) the lowest and at 300μg/mL the EA (-12.881 ± 0.018%) and DCM (-0.847 ± 0.009%) extracts displayed inhibition.

An attempt was made to isolate and elucidate the compounds in the extracts but unsuccessful, although the TLC results indicated several compounds that can be elucidated in future study.

Keywords

Hippobromus Pauciflorus, Pentanisia prunelloides, Lipid peroxidation, anti-oxidants.

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List of Abbreviations

4-HNE = 4-Hydroxyalkenal

A = Absorbance

AAE = Ascorbic acid equivalent

ABTS = 2,2'azino-bis (3-ethylbenthiazoline-6-sulphonic) acid

ANOVA: Analysis of Variances

$AlCl_3$ = Aluminium chloride

BHT = Butylated hydroxytoluene

C = Concentration

DCM = Dichloromethane

dH_2O = Distilled water

DNA = Deoxyribonucleic acid

DPPH = 2, 2-diphenyl-1-picrylhydrazyl

EA = Ethyl acetate

Etc. = Etcetera

EtOH = Ethanol

F.R. = Free Radical

FRAP = Ferric Reduction Anti-oxidant Power

$FeCL_3$ = Iron Chloride

$FeSO_4$ =Iron sulphate

g = Gram

µg = Microgram

GAE = Gallic acid equivalent

H_2O_2 = Hydrogen Peroxide

***H* =** *Hippobromus*

HCl = Hydrochloric acid

HOCl = Hypochlorus acid

IUCN = International Union for Conservation of Nature

n-Hex = n-Hexane

L.P. = Lipid peroxidation

MDA = Malondialdehyde

MeOH = Methanol

MIC = Minimum Inhibitory Concentration

Mins = Minutes

mL = Millilitre

mM = Millimetre

mg = Milligram

n = Non-bonding orbital

Na_2CO_3 = Sodium Carbonate

nm = Nanometer

***P* =** *Pentanisia*

π = pi bonding orbital

π* = pi anti-bonding orbital

PUFA = Polyunsaturated Fatty acid

QE = Quercetin equivalent

ROS = Reactive Oxygen Species

σ = Sigma bonding orbital

σ* = Sigma anti-bonding orbital

SA = South Africa

TBA = Thiobarbituric acid

TBARS = Thiobarbituric acid reactive species

TCA = Trichloroacetic acid

TLC = Thin layer Chromatography

U.S. = United States

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Chapter 1

1.1 Introduction

Throughout modern civilisation, there has been a conviction that old-fashioned practices of using plants and plant-based extracts for the treatment of ailments are old and ineffective while this has not held out to be true because of the need for new medications to treat and cope with emerging and re-emerging diseases (Amuka, 2013).

People who are considered to be of the older generation who possess knowledge and information about traditional medicines are fast disappearing. It is therefore very important that such knowledge be collected and stored for future generations using modern technologies and through the migration from traditional medicines to modern (western) medication.

It is estimated that only one percent of medicinal plants recognised by scientists that are acknowledged for industrial purposes due to a large amount of potential medication being rejected at some stage of testing before final use by the public.

An enormous amount of drugs or drug components have been discovered with the help of ethnobotanical knowledge of past traditional uses of plants by indigenous civilisation of the region (Amuka, 2013).

A substantial number of beneficial compounds are still to be discovered and documented owing to the variety of plant species around the world with literature reporting that hardly two percent of the earth's natural plant resources are being exploited and harnessed for pharmaceutical purposes.

A primary reason that plants are chosen as a potential source of new medicines is that they are readily available to researchers and the public either free of charge or at low cost. This explains why most rural communities especially in developing countries depend on medicinal plants to maintain health and wealth.

In the last three decades of the twentieth century, the scientific assessment of medicinal plants and their effectiveness in treating diseases has garnered immense attraction in the scientific society. With the advances of scientific approaches of scrutiny, countless medicinal plants have come under scientific scrutiny which has led to the revelation of their bio-active principles (Amuka, 2013).

Although it is feared that once the production costs to fulfil the demand will always be a decisive factor as to which products reach the world markets. A variety of under-used crops and other plant sources are acknowledged and reported to be available to fulfil the demands of both the developed and developing countries.

Throughout the times, wild or cultivated plants have been brought into a new habitat or society to meet a growing need for certain quantity and quality of product whether it is used as food and/or as an industrial product (Alexaides, 2004).

If the needs cannot be achieved via natural resources, organisations have turned to the development and improvement in recent years of synthetic manufacturing techniques to produce quality synthetic alternatives for pharmaceutical companies to fulfil the demands (Alexaides, 2004).

With growing importance that is placed on information-based approaches and with subsequent advancement in fields like chaos and complexity theory, fuzzy logic, computer modelling, robotics, molecular biology, bioinformatics and genomics all at least point to an unparalleled capability to record and store information and environmental information, indicating that in the twenty-first century, people now more than ever, have the ability to readily access that information.

Technological means of communication such as social media, smart phones, mobile applications, online references, etc. have increased the ability for people to be able to gather, share, store, manipulate (which can be a double-edged sword) and communicate data and information, thus technological advancement has qualitatively changed the meaning, use, value and distribution of information with the world.

Information itself has become a central organising idea in which science and technology, governmental departments as well as in business and corporate organisations in order to venture into profitable activities.

Knowledge and information are not simply terms that can be used only as a means of converting natural resources into commodities, rather knowledge itself is now viewed as a commodity that can be or is sold and/or exchanged according to set formal rules that has attributed specific ownership, rights and formal access to that information and who benefits from endeavours that may possibly arise from its shared use.

Due to this shift in the valuation of knowledge and information, cultural and biological diversities of knowledge due to the abundance and variations of indigenous knowledge, knowledge itself has adopted an entirely new and very significant meaning for indigenous people.

The movement of these ethnic resources and cultural knowledge into different spheres of the global markets and global economy inevitably leads to its transformation as certain types of information and genetic resources become increasingly valued as commodities.

It has been reported that there are growing concerns among some indigenous leaders that promoting the giving out of traditional knowledge can lead to its misappropriation and eventual commodification by third parties that can lead to use by the third parties without compensation to the bearers of the original knowledge.

For researchers a crucial factor about information is that it is typically very costly to produce but very cheap to reproduce (Alexaides, 2004).

Ethnobiologists have begun to experience a catch-22 situation between their responsibility towards their peers and funders who expect free exchange of knowledge and also their responsibility to their indigenous collaborators who may limit the amount of knowledge they share with researcher leading hindrances on the depth of knowledge that can be documented about the natural resources (Wickens, 2001).

Academic publications, whether online or hard copy, are a perfect illustration of this as they are critical for the establishment of scientific credibility, consensus and professional advancement but inadvertently transform the legal status of indigenous knowledge which leads to the compromise of the proprietary rights and control of the indigenous group over their shared knowledge.

Although the connected increase in the flow of information can create new opportunities for scientific advancement, development and innovations; it can also be this appropriation of knowledge by different interest groups that has created new forms of exclusions that are fuelling the already growing tensions between stakeholders with diverse claims, needs and expectations of this knowledge (Wickens, 2001).

The term information refers to data that can be codified in some explicit form of documentation while knowledge is said to reside on the user's subjective context of action based on that information.

In other words knowledge resides in the customs people speak and interact with the environment and each other with the flexibility of the knowledge inherent in linguistic behaviour and can be stable or in a state of change.

Knowledge may be accessible to people for sharing in the ways of public beliefs shared by the members of the community and it may exist in special anointed individuals, it is also said to exist inside of them by means of general rules that are learned by public consensus or as an ability to treat new situations in an imaginative way and that is to say that knowledge is living, dynamic and socially produced.

Some forms of knowledge can be made explicit and written down for storage as publications made available to the public in libraries, academic publication, etc. but much is unspoken i.e. most "juicy" information is non-codable and can only be transferred indirectly or through non-verbal human interaction.

Reducing indigenous knowledge to a cumulative pool of information objectifies cultures and hinders the essential and creative aspect of indigenous people continually re-inventing themselves due to modernising environment and production of new knowledge in the face of change.

In theory the sustainable productivity and utilisation of natural resources (i.e. medicinal plants) are feasibly realistic even though in practice it has become economically unviable because that is the standards of living in developed countries continue to grow higher.

In order, to meet this demand, cheap imports are required from less developed or developing nations on their terms irrespective of the human and environmental concerns in the developing nations.

In theory it is plausible to feed the world's population but today's political and economic climate render such a possibility virtually implausible (Wickens, 2001).

Plants that originally come from Africa make up 8% of the 1100 medicinal plants that are commercialised and utilised globally whilst only a scarce computable medicinal marketplace research have been carried out in Benin, Gabon, South Africa (SA), Ghana and Tanzania in Africa have highlighted the high risk associated with over-exploitation of medicinal plants of worldwide importance of African medicinal crops dating back to the invasion of the earliest European colonisers (Moyo, Aremu & Van Staden, 2015).

World trade and world markets today are progressively in the control of multinational corporations that are enthused by making a profit.

This fact is illustrated by these corporations creating genetically modified plants which possess seeds that only react to the multinational corporation's brand of pesticides/ herbicides however now the seeds may have a terminator gene that is introduced in the crop to obstruct the germination of future generations of plants thus confining farmers to purchase procure new seeds annually from the multinational corporations.

Food security will continue to be one of the world's foremost problems more so for the less developed nations where debilitating debt repayments due to a variety of economic factors such as low agricultural inputs which includes soil erosion, habitat desertification and deforestation make it hard for the countries to be economically strong. But world trade makes it almost impractical to grow ample sustenance for the needs of the world's population.

It has been documented that throughout history that there has been a vast amount of wild plants have been taken from there indigenous habitat and brought into foreign habitats to aid in curbing an ever increasing requirement for guaranteed quantity and quality of healthy food

These nations are then regrettably obliged to export non-essential goods to developed countries in order to obtain foreign currencies.

Then again, developing nations do possess the ability to supply food for export to less developed nations but knowingly or unknowingly forsake to do as such due to the reason that crop excesses will lead to lower values for their farmers which then would result in lower standards of living.

But even if hypothetically, there were to be surplus food made available to the nations in need there would be "hindrances" that would may doing so difficult (Wickens, 2001).

Also it is vital for countries to conduct a full assessment of the conservation status of their flora and according to Moyo et al (2015) to date SA is one of a few countries globally that have evaluated the status of their whole vegetation utilising IUCN red List Categories but with additional categories including the categories of critically rare, Rare and declining.

Today people live in an ever increasingly "interconnected" and "interdependent" world that is viewed more than ever as a shared social space i.e. it is a place that is moulded by transnational ecological, economic, legal, political and technological ties and the prominence of capitalism in the 16th century presupposed the creation of the world economic system.

Globalisation of medicinal plants has progressively subjected indigenous plant resources to over-exploitation which can result in the elimination of vital plant species as a majority of most medicinal plants are locally-derived but they are used globally (Moyo, Aremu & Van Staden, 2015).

Globalisation can be best understood as a set of related but multifaceted and differentiated political, economic, social and cultural processes that is characterised by increased world trade also with an increased international flow of capital that is associated with the growth, expansion and consolidation of transnational corporations (Alexaides, 2004).

Illustrated by the fact that 400 multinational and transnational companies were reported to be responsible for more than half of the total world output and of particular importance and concern to ethnobotany and ethnobotanists, is the increased ability for food, agriculture and

biotechnology multinationals to shape the future production, marketing and consumption of agricultural and food products (Alexaides, 2004).

Globalisation is also characterised by the global reorganisation of production exemplified in the international division of labour and structure and with this knowledge allowing companies to adapt to rapid changes in the market where they can exploit specialist niche efforts to form a strangle hold on the market and can include de-industrialisation of advanced societies.

Changes toward globalisation are connected with a shift in power between the private and public sector leading to wide scale privatisation and deregulation of many sectors of the economy which may include transportation, communications, education, health and science.

Increasing interdependence between nations does not imply increasing homogeneity or equality between nations and regions. On the contrary; evidence suggests that globalisation has increased social and economic inequalities (Alexaides, 2004).

According to world-systems theories, they suggest that from the beginning that capitalism created a global market and global divisions of labour characterised by "core", "periphery" and "semi-periphery" zones and that the expansion of capitalism has always depended on the unequal exchange between core and periphery i.e. the haves and the have-nots.

Peripheries tend to be politically and economically marginalised regions with limited access to the manufactured goods and the services produced at the centre but with high biodiversity and diversified resources management systems.

Cores are referred to as such because they accumulate political and economic power and are deficient in certain resources and depend on the flow of these materials from the peripheries to sustain their growth. There are three vital aspects to the relationship between centres and peripheries.

Firstly, there is a historically consistent pattern in the relationship between centre and peripheries when it comes to resources extraction which is exemplified by natural resources from peripheries acquiring value for centres as they assume control over the resources.

Thus displacing the communities that traditionally controlled them and typically leading to the overexploiting of the resource and eventually, where possible and affordable leading to the developing cheaper alternatives or more relocation to "convenient" areas of production.

Secondly, the structural asymmetry that exists between peripheries and centres make it extremely difficult for the natural resources to flow in ways that serve to strengthen the periphery.

Thirdly the structural asymmetry between peripheries extends from the local to the international scale leading to the unequal shares of natural and manufactured goods (Barrister & Barrell, 2004).

While the nation-state is clearly a vital stakeholder with respect to proprietary claims on genetic resources, other stakeholders, such as local communities and indigenous tribes within nations have claims that to date have not received the same level of legal recognition as nation-states. Finally globalisation today is characterised by the growth of the service and knowledge sector.

Information has become a cornerstone of international economic growth and of corporate and social organisations with the interdependence between capitalism and industry in the nineteenth century having yielded to an interdependence of capitalism and information where information has replaced production as the primary source of wealth and power today.

The role of that information plays in today's corporate world is shown by the cutting-edge business concept of knowledge management which seeks to optimise how knowledge is produced, stored and managed and thus allowing companies "to react with strategic agility, sensing and responding quickly and innovatively shape the global market opportunities presented to the companies" this allows the companies a competitive advantage in an increasingly unpredictable and dynamic business environment (Alexaides, 2004).

Process of commodification has accelerated in later years due to the growth of biotechnology and knowledge based industries and also supplemented with the drastic improvements in communications and data-processing technologies while trade secrets, copyrights and patents are examples of institutionalised mechanisms used by corporations and individual people to privatise information.

While attempting to guarantee economic returns that can occur to ensure the continued production and the swift upsurge in the number of patents filed in the United States patent office is another indication of the growing economic importance of knowledge-based technology.

Questions arising due to property rights, cultural and genetic resources are immensely complex they are rapidly evolving while the controversies surrounding these issues signal the polarisation of the social and political environment through which genetic resources flow as they move through the global economy.

Conflicts between diverse actors are heightened given that it is often difficult to ascribe clear geographical cultural boundaries to the distribution of many of these resources and that different stakeholders may have competing claims over these resources (Alexaides, 2004).

1.2 Medicinal Plants

Medicinal plants are regarded as plant and/or plant material that is utilised byman and anima for uses such as traditional medicines, phyto-pharmaceuticals, new drugs, galencials and herbal tisanes, intermediates for drugs in manufacturing industry.

While medicinal plants are also being used as pharmacological goods and health foods. Medicinal plants have applications in pharmacy, pharmacology, pharmacognosy, naturopathy, aromatherapy and homeopathy.

Pharmacy is the term that is attributed to the discipline of the applied expertise of formulating and compounding of medicines or the dwelling where medications are distributed while pharmacology is the discipline of drug medication which focuses on the understanding of drug configuration, usages, effects and modes of action (Wickens, 2001).

Pharmacognosy is the division of pharmacology which focuses on crude natural drugs including medicinal crops, while aromatherapy is a class of holistic treatment where ailments are remedied with the use of massage techniques with aromatic oils which apart from their pleasant fragrant smell they are said to have anti-inflammatory, anti-bacterial, stimulatory, antispasmodic, anti-fermentative, hormonal and cicatrizant properties.

Naturopathy is a therapeutic practice that fully depends on natural therapies for example clean, natural, fresh air, massage, organic foods and sunlight to treat body ailments. Homeopathy is a part of medicinal therapy that has its basis on the tenet of using small amounts of the remedies that would in vast volumes produce effect identical to those of the diseases that are being treated to acquire enhanced understanding of the effects.

The earliest transcribed medicinal crop records should be the Samerian cuneiform writings on Mesopotamian clay tablets which have been dated to the time of 3400 BC. But there is further evidence of signs that trepan surgery had been carried out during the Neolithic era which is dated to the time between 5100 and 4900 BC, suggesting there could be earlier knowledge of the application of medicinal plants as anaesthetics or intoxicants or antibiotics.

From Ancient Egypt, three medicinal papyri that have been discovered are dated to the time around 1550 BC and 1500 BC respectively. The Ebers papyrus provides knowledge of 842 prescriptions that have 328 different ingredients that have no found applications in sorcery (Wickens, 2001).

The primary reason of plant life is the generation of a suitable and sustainable natural habitat for every existing organism that are present in that environment, the present population expansion and increasing demands on the environment and lifestyles of humans' for ever increasing living standards in every nation has consigned a virtually unattainable environmental load.

Therefore there is a perpetually climbing inevitability to help the ecosystem to cope and maintain the prevailing genetic diversity for use by impending generations (Alexaides, 2004).

Human reliance on medicinal plants has been well documented in academic research and through traditional knowledge and has been used for both humans and animals ever since the dawn of time.

In plants, there are compounds known as secondary metabolites and they have physical and economic benefits for those who are invested in human and animal health. The knowledge of plants and their medicinal properties has been an important aspect for human and animal survival that people who specialize in the knowledge and practice of it can make a living from the cultivation to manufacture, sales and use of medicinal plants and plant derivatives.

Knowledge of medicinal plants was and still is a vital part of South African and African knowledge as the knowledge is passed on from one generation by word of mouth though little has been documented. There have been documented cases of medicinal knowledge having being recorded in text such as the Ancient Egyptian and Chinese civilisations as pointed out earlier and also other cultures (Moyo, Aremu & Van Staden, 2015).

Also the on-going loss of ethnobotanical knowledge due to little or no records of the knowledge occurring, most writers have stressed that the over-harvesting of medicinal material from their natural habitats is a leading threat to the safeguarding of traditional medicines (Moyo, Aremu & Van Staden, 2015).

The global trade of medicinal plants is a large and possibly lucrative industry with large volumes of products being imported and exported. More than 70000 tonnes of plant substance is used in SA annually with estimates of 134000 income-earning chances that can be generated by the formal and informal trade of medicinal plants and related products through numerous markets throughout SA especially during the summer-rainfall season.

A lot of plant material that is cultivated and provided is usually found mostly from the gathering of wild plant resources that has led to alarm over the sustainability of the plants and threats to the resources are made worse due to habitat transformation such as forest destruction or the tarring or paving of the roads occurring over the world due to modernisation with some vegetation types becoming more harmfully distressed than others.

With the increase of medication needed to aid in dealing with existing or new diseases, people and science have turned to medicinal crops as a source of novel medications or templates for the manufacture of new drugs.

The populace of SA continues to increase in the middle of an HIV/Aids pandemic and other ailments such as diabetes, oxidative stress-related diseases and an increase in the life expectancy people nowadays requires the widespread use of herbal treatment of opportunistic infections coupled with existing medications and the need for medicinal plant supplies is rising to stay abreast with this increased demand.

A wide range of species around the world are displaying marks of unsustainable reaping with the sizes of the exchanged components (for instance bulbs and roots) declining while distances that are travelled to harvesting source increasing as it becomes harder to locate the material and thus the supply becoming increasingly irregular which leads to higher pricing of plant material and some plants eventually becoming unavailable in certain markets due to the over-exploitation (Williams, Victor & Crouch, 2012).

While there is an unavailability of supporting empirical information, the statement that “80% of the continent’s population depends on the herbal medicine for their primary health care” is intertwined with African ethnopharmacological and ethnobotanical literatures and is used as justification for ethnobotanical research.

Researchers and policy makers still use this and other out-dated estimates for the reason that the latest measurable records on usage of medicinal crops does not exist or if it does, it does not encompass all plants that are used as medicinal plants but it is indisputable that medicinal plants have performed an elemental function in primary health care in Europe, Africa, Asia and other continents for eons.

Due to this highlighted rise in need of medicinal plants both locally and globally, it is of importance for countries that contain medicinal plants and other plants in general to evaluate the preservation status of their plant life utilising standard IUCN Redlist categories.

Up till now, SA is the single nation that has evaluated the threat statuses of its guidelines of using the categories of extinct, threatened, least concern but containing added categories which are declining, rare and critically rare (Williams, Victor & Crouch, 2012).

Medicinal market quantifiable reviews can offer a reliable basis of information on the amount of exchanged plant species and reduces the estimated amount of wild plant population reduction by having knowledge of the plant leading to its sustainable harvesting (Williams, Victor & Crouch, 2012).

Moyo et al (2015) reports that in an examination of medicinal geophytes that are added to South African medicinal markets and reported on a survey by Williams et al in 2007 proved that information relating to the magnitude of bulbs that are traded in the market had meaningfully decreased between 1995 and 2001.

Studies like those of Williams et al (2007) can produce prised statistics on the extent of dealings if medicinal plants threatened by overharvesting, which in turn can add to species-specific conservative measures.

The fertility of medicinal crop resources in Africa springs from the massive floral assortment that can be located throughout the continent principally in the biodiversity hotspots of the continent.

In African regions such as the Cape Floristic Region, Pondoland Succulent Karoo, Maputaland, Albany, West Africa, the Eastern Afromontane, Madagascar and the Indian Ocean Islands, the Guinean Forest and coastal forest of Eastern Africa are characterised by their distinctive plant abundance and vast amounts of endemism of plants species that are found there (Moyo et al, 2015).

The rush for medicinal plants for the market and utilisation has resulted in the documentation of them in various African nations for numerous years and the decline in bulb size and other exchanged crop parts has led to unpredictable and unreliable supply of certain plants and/or plant parts at medicinal markets.

The scarce quantifiable medicinal marketplace analyses that have been conducted in Gabon, Benin, SA, Sierra Leone and Tanzania have emphasised the dangers presented by over-exploitation of medicinal crops and in history there have been early attempts that encouraged the sustainable collection, preservation and extensive ethnobotanical observations of medicinal crops.

The continuous, gradual dwindling of ethnobotanical knowledge and growing destruction of natural environments on the continent continues to deplete the richness of medicinal plants found thereby restraining the possibilities for novel drug discoveries.

The sustainability of medicinal plants is heavily subjective to the type of collection of the plants, the relative richness and growth rates. The vulnerability of medicinal plant species to over-harvesting remains a function of life forms and plant parts utilised.

Conservation concerns of natural resources more so medicinal plant species to this day even with advanced technologies remains a global challenge and unfortunately the rate of the loss of plant biodiversity is estimated to grow over time (Moyo et al, 2015).

Moyo et al (2015) report that the harshness of this dilemma is pointed out by the lofty quantity of plant species presently under strain (i.e. they fall under the categories of critical endangered, endangered or vulnerable in the IUCN Redlist).

Cameroon for example has 54% of its vegetation under strain; Tanzania has 46%, whereas Ghana and Nigeria have 36% under strain. The DRC and Cote d'Ivore have 33% under strain and Kenya has 29% (Moyo et al, 2015).

The common reduction in plant diversity is frequently accredited to a number of aspects like habitat destruction, introduction of alien species to the environment that causes an increase in competition for nutrients, mortality from introduced diseases, over-exploitation and pollution for a range of functions including medicinal purposes, food and shelter.

Generally, the worth of medicinal plants is basically intimate, due to their major involvement in the fields of cultural identity and livelihood, financial income and healthcare. In Africa it is predicted that 9% of the 60000 plant taxa that have been documented possess over 16300 therapeutic uses.

The African medicinal plant industry recently has come under the spotlight due to concerns regarding the sustainable usage of wild medicinal crops, for they are the usually the central resource of traditional medications and bulk used industrially. A concise, accurate and up to date data with regards to plant species sold, their costs and the volume used in the markets are the initial actions in identifying species with concerted efforts regarding the conservation and resource management priorities (Moyo et al, 2015).

However with the shortage and sometimes the absence of scientific data and literature to the public on these vital characteristics of medicinal plant trade in Africa is mutual and continues to be a key obstacle and with the ever growing acknowledgement of the prominence and worth of such information, fresh quantitative studies assessing the quantities of medicinal crops transacted have been reported for a minority of African countries like SA, Gabon, Tanzania and Sierra Leone for instance.

Information that can be obtained from these surveys will undoubtedly add positively towards the estimation of the conservation standing of the frequently and infrequently utilised medicinal plant species and as well as other plant species.

In South Africa, it is reported that an approximated 85% of the medicinal plants cultivated locally utilised are mainly made up of the non-renewable parts of plants like bulbs, rhizomes and bark. It is not only SA that non-renewable parts are sold in the market; other countries have been reported to be using similar levels of non-renewable parts in the market as well such as, Gabon, Sierra Leone and Ghana (Moyo et al, 2015).

Just like any species of plant that is needed in nature for organisms to survive, the removal of essential parts such as wood bark, bulbs, corns, roots or the whole plant itself will typically lead to the death of a species more so excessive utilisation will definitely lead to destruction of a species.

The harvesting of leaves, fruits and seeds is regarded less detrimental to plant survival but excessive cumulative harvesting of leaves, fruits or seeds may eventually affect the reproduction of such species both in the market and for its survival can lead to difficulties that affect the rates of growth, survival and reproduction of the species (Moyo et al, 2015).

Also there will be complications with the population structure and dynamics of the environment that the plants inhabit including the community structure and arrangement i.e. plant–plant and plant-animal dynamics and relations which affect the nutrients and organic matter forces at work, energy exchange available to ensure plant survival (Moyo et al, 2015).

1.3 Lipid Peroxidation (L.P.)

Three separate steps are involved in L.P.: initiation, propagation and termination. The process is reported to be the key molecular mechanism encompassed in the toxicity process which leads to the fatality of a cell; it is a very intricate activity that occurs in both animals and plants (Gaschler & Stockwell, 2017; Repetto, Semprine & Boveris, 2012).

This complicated process might include the initiation and proliferation of lipid radicals, the a bsorption of an oxygen atom, a double bond rearrangement in unsaturated lipids and the consequent consequence of reactions resulting in damage to membrane lipids and the development of a range of breakdown products that may include compounds such as alcohols , alkanes, ketones, esters and aldehydes.

Lipid peroxidation is a chain reaction that happens easily on unsaturated fatty acids initiated either by the abstraction of a hydrogen atom or by the addition of radical oxygen resulting in oxidative damage to polyunsaturated fatty acids (PUFA's), since PUFA's are more vulnerable to oxidative damage than saturated fatty acids death (Gaschler & Stockwell, 2017; Repetto, Semprine & Boveris, 2012).

It is suggested that the activated methylene group renders the methylene carbon-hydrogen (C-H) bonds weaker than the carbon-carbon double bond (C=C), also the electronegativity of the elements plays a factor and thus the hydrogen of the methylene group is more vulnerable to abstraction.

This results in a PUFA with an unpaired electron on the carbon-centred radical that is followed by the molecular rearrangement of the double bonds to form a conjugated diene that helps to stabilise the radical.

The conjugated diene then combines with molecular oxygen to form a radical lipid peroxyl. The formation of peroxyl radical leads to the production of organic hydroperoxides, which ca n in turn, remove hydrogen from another PUFA.

Then the peroxyl radical is also able to react with a nearby PUFA to stabilises itself thus propagating the oxidative damage to other PUFA’s. This reaction is termed propagation and can yield substances such as alkyls, peroxyls and alkoxyl radicals are produced in the F.R. chain reaction.

Reduced Iron complexes (Fe^{2+}) can also bond with lipid peroxides (ROOH) to yield alkoxyl radicals while oxidised Iron complexes (Fe^{3+}), which bond a lot slower than Fe^{2+} complexes, produces peroxyl radicals.

Both radicals are then able to participate in the propagation of this chain reaction. In the presence of transition metal ions, lipid hydroperoxides are able to yield radicals that are able to re-initiate L.P. by means of redox-cycling of these metal ions death.

Transition metals produce L.P. by stimulating the oxidative ability of hydrogen peroxide by advancing F.R. mediated processes and other accepted mechanisms, thus transition metals are able to bind onto negatively charged phospholipids in the membrane bilayer which change the physical characteristics of the bilayer and favour the propagation and initiation reactions of L.P (Gaschler & Stockwell, 2017; Repetto, Semprine & Boveris, 2012).

Transition metals, such as Fe^{2+} and Cu^{2+}, stimulate L.P. by the reductive cleavage of endogenous lipid peroxides of membrane phospholipids to create an alkoxyl and a peroxyl radical.

This complicated metal ion-catalysed breakdown of lipid hydroperoxides may include end products such as cytotoxic aldehydes and hydrocarbon gases such as ethane, methane, etc . (Gaschler & Stockwell, 2017; Repetto, Semprine & Boveris, 2012).

The F.R. chain reaction continues until two F.R.s react together to cease the chain reaction. The reaction could as well be halted by a chain breaking anti-oxidant for instance Vitamin E (α-tocopherol), which acts as excellent proton donor producing lipid peroxides and α-tocopherol phenoxyl radical which is stable and will not act as a F.R. although it is a radical. The above process is referred to as a dismutation.

Lipid peroxidation causes a decrease in the fluidity of a bilipid membrane of organisms and also affects the barrier functions of the membrane. The numerous products of L.P. for instance hydroperoxides or their aldehyde offshoots hinder blood macrophages actions, protein synthesis and change enzyme activity and chemotactic signals.

There are many molecular biological mechanisms that produce L.P. in biological systems, it is reported that L.P. might be the result of the production of intermediates of oxygen that is partially reduced (i.e. the homolysis of hydrogen peroxide and hydroxyl radical production).

Or as a result of direct auto-oxidation of lipids, which is a biological process that happens due to the homolysis of hydroperoxides caused by the scission of the lipid hydroperoxides to produce a peroxyl and alkoxyl radical and the production as intermediates of nitric oxide metabolism while also resulting from modifications of lipid membrane surface structure.

Lipid peroxidation results in the breakdown of lipids and a varied array of products for instance conjugated dienes of lipid hydroperoxides and ancillary products which include ethane, hexane or pentane or DNA and F2-isoprostane.

The assessment of these conjugated dienes is of interest since they identify the molecular reorganisation of PUFA’s through the initial phase of L.P (Gaschler & Stockwell, 2017; Repetto, Semprine & Boveris, 2012).

Lipid hydroperoxide is an additional indication of initial reaction of F.R.s and is a certain type of damage of cells and the other products are frequently utilised to evaluate oxidative stress.

Among the many degradation products of L.P., lipid peroxides are aldehydes for example MDA and hydrocarbons for example ethylene and ethane which are generally assessed end products of L.P. in the laboratory death .During the peroxidation process there are many reaction intermediates that are produced such as aldehydes for instance malondialdehyde and 4-hydroxynonenal, ethane, isoprostains, cholesteroloxides, pentane, and 2,3-transconjugated dienes.

The biological actions of malondialdehyde and further aldehydes that are end-products of lipid peroxidation involve the cross-linking with proteins and DNA, that changes the composition and proper purpose of molecules and malondialdehyde and 4-hydroxynonenal have been reported to display tissue toxicity (Gaschler & Stockwell, 2017; Repetto, Semprine & Boveris, 2012).

Another complication with the aldehydes is that aldehydes are more diffusible than F.R.s, which can cause impairment to other distance sites however under normal conditions aldehydes are fairly readily eliminated from cells by certain enzymes to manage their metabolism and negative effects they can impart if they occur in excess levels.

Lipid peroxidation similarly participates in the controlled death of a cell that naturally occurs in malfunctioning and/or old cells. 4-hydroxynonenal (4-HNE) is reported to have to stimulate apoptosis in particular circumstances and literatures have likewise acknowledged that L.P. may act as the principal driver of ferroptosis which is a form of controlled necrotic cell death (Gaschler & Stockwell, 2017; Repetto, Semprine & Boveris, 2012).

1.4 Anti-oxidants

The total anti-oxidant capacity of an analyte is reported as the collective capacity of an analyte to scavenge F.R.s; this is useful for health purposes because plants contain a variety of anti-oxidants (El-Aal, 2012).

The information and knowledge of total anti-oxidant capacity is important in organisms maintaining a reasonable level of health.

Anti-oxidants are naturally occurring substances that fight oxidative damage that may occur to and/or in biological entities, this is done by the anti-oxidant decelerating or thwarting the oxidation process or processes which causes cell damage.

This process is achieved by the anti-oxidant being oxidised instead of the cells and by subduing rogue and unstable F.R.s by donating electrons to those unstable F.R.s.

The F.R. is then stabilised by the donation of the electron which is then converted to a harmless compound which is then dispelled from the body. Anti-oxidants work in harmony with other anti-oxidants.

Anti-oxidants are either hydrophilic or hydrophobic in nature where the hydrophilic anti-oxidants are reported to be active in blood plasma while hydrophobic anti-oxidants are said to protect the cell membrane (El-Aal, 2012).

Anti-oxidants are separated into two classifications on the basis of how the anti-oxidant operates: preventive or chain-breaking anti-oxidants.

Chain breaking anti-oxidants for instance Vitamin E and Vitamin C stop the progression of radical formation by stabilising the F.R. thus the F.R. is stopped from inducing further damage via the chain-like process of radical formation.

Preventive anti-oxidants like catalase and superoxide dismutase (SOD) avert radical chain initiations by means of scavenging for radicals that are initiators of the reactions where they are stabilised by the anti-oxidants and these anti-oxidants are also able to stabilise metals such as Iron and Copper which can act as metal radicals in the body.

Anti-oxidants are available to humans mainly through vitamins, enzymes and minerals. Trace elements such as Selenium, Manganese and Zinc are of great importance as they are constituents of many anti-oxidant enzymes for instance SOD, glutathione peroxidase and catalase (El-Aal, 2012).

Vitamin E is made up of a group of eight tocopherols that is lipid-soluble and its function in the body is to provide protection to cell membranes which are generally made up of fatty acids (El-Aal, 2012).

Vitamin C is a vitamin that is water-soluble which scavenges for F.R.s which are found in aqueous surroundings inside the body and the general consensus amongst a majority of nutritionists agrees that natural food is the optimum source of anti-oxidants.

There is a large range of anti-oxidants that are active in the body which may either be enzymatic or non-enzymatic anti-oxidants (El-Aal, 2012).

Non-enzymatic anti-oxidants comprises of low molecular weight molecules like coenzyme Q10, Vitamin E, β-carotene, reduced glutathione Vitamin C, cysteine, lycopene, melatonin, Lutein, Vitamin B2, thiols, flavonoids and micronutrients.

Enzymatic anti-oxidants include glutathione, SOD, glutathione reductase, catalase, glutathione peroxidase and glutathione-s-transferase to list a few examples (El-Aal, 2012).

1.5 Oxidative stress and F.R.s

The most commonly produced Reactive Oxidative Species (ROS) in humans are hydrogen peroxide (H_2O_2) and superoxide anion that are able to damage biochemical molecules including nucleic acids, cells, cell membranes and amino acids to name a few examples (El-Aal, 2012).

Exposure of ROS to proteins leads to the denaturation of the proteins. It also results in the loss of proper protein function, fermentation, cross-linking and aggregation of connective tissues for example collagen but more so the highly detrimental effect of ROS is the initiation of lipid peroxidation.

There are a variety of ROS that are reported to be accountable for biological oxygen toxicity in organisms. These oxygen reactive species include intermediates caused by the partial reduction of oxygen reactions for instance H_2O_2, nitric oxide, hydroxyl radical, superoxide anion, peroxinitrite, peroxyl radical and singlet oxygen while reactive aldehydes lipid peroxides, Hypochlorus acid (HOCl) and hydroxyl radical are regarded to be oxidants that play a critical part in the vascular biology of organisms (El-Aal, 2012).

Oxidative stress is defined as a condition of tissue oxidation that is unbalanced; that is a state in which the tissue cells are exposed to extreme levels of molecular oxygen or its chemical derivatives termed Reactive Oxygen Species (ROS).

This can occur because oxygen is a main oxidant in metabolic reactions in biological entities which is used to generate energy due to various organic molecules that have undergone oxidation under normal physiological conditions.

Molecular oxygen can be subjected to a number of reactions that generally leads in the formation of water, H_2O_2 and oxygen radical.

Oxidative stress occurs due to metabolic reactions that utilise oxygen and is said to be the disturbance of the equipoise of anti-oxidant and pro-oxidant systems in undamaged and healthy cells (El-Aal, 2012).

This shows that undamaged cells contain an anti-oxidant and pro-oxidant system that constantly produce and cleanse the undamaged cell of generated and/or already present oxidants during normal aerobic metabolism (El-Aal, 2012).

When this excessive oxidative happens, the pro-oxidant system of the cell outbalances the anti-oxidant system of the cell which can lead to oxidative harm to nucleic acids, carbohydrates¸ lipids and proteins that lead to the death of the cell in cases of severe oxidative stress.

Chronic and mild oxidative stress can change the anti-oxidant system in the cell either by creating or suppressing proteins that are involved in the anti-oxidant system thus diminishing cellular reserves of anti-oxidants for instance Vitamin E and Glutathione.

Oxidative stress is documented to participate in the processes of aging and documented to be involved in a variety of chronic diseases for instance coronary heart disease, diabetes, cancer, obesity, type2 diabetes, atherosclerosis, hypertension and cataracts to list a few diseases.

Fruits, vegetables and less processed food are documented as foods that are rich in anti-oxidants such Vitamin C, carotenoids, polyphenols, etc. that are associated with a reduction of the dangers of chronic diseases. (El-Aal, 2012)

Several assays; like FRAP, DPPH and ABTS are utilised to assess the "total" anti-oxidant capacity of analytes and have been established to scrutinise the contribution of oxidative stress in cellular environments to assess the bioavailability of functional anti-oxidants that may be present.

So as a consequence, anti-oxidants that are water-soluble like uric acid, protein thiols and ascorbic acid have been attributed to be of main influence on these assays while lipid-soluble anti-oxidants like tocopherol and carotenoids demonstrate little influence over many assays.

Free radicals (F.R.s) and other reactive species are understood to perform a pivotal function in oxidative stress that is involved in numerous human diseases, so ascertaining their exact function in the damage necessitates the capability to assess them and the extent of oxidative damage F.R.s and other reactive species can bring about.

Revelations into the mechanisms of actions of the anti-oxidants and their true anti-oxidant capacity may result in suitable and improved approaches being developed to optimise the anti-oxidant defence in organisms.

F.R.s aid the immune system by imparting positive effects on it while they can also have detrimental effects by damaging DNA, lipids or proteins and are customarily occur in small concentrations.

There are two primary sources of free radicals, i.e. endogenous and exogenous sources (El-Aal, 2012).

Endogenous sources are created inside the cells. The F.R.s act inside the cell but can also be discharged, when necessary, into the nearby region for involvement in biological processes. They can be a consequence of auto-oxidation reactions, following the inactivation of small molecules like Flavins and reduced thiols or as a consequence of the biological activities of particular oxidases, dehydrogenases, cyclo-oxigenase, lipoxygenase and peroxidases (El-Aal, 2012).

Exogenous sources are any source of F.R.s that are not produced by the body and they include ionising radiation, industrial pollutants, excessive exposure to the sun and cosmic rays and as well as heavy metals, ozone, unsaturated fats, cigarette smoke, alcohol, medicinal x-rays, nitrous oxide and pollutants that are found in food, water and air.

Production of F.R.s happens in a number of ways, they can be formed when an organism is exposed to ionising radiation whilst humans can produce F.R.s through physical exercise, which can cause interruptions of the homeostatic equilibrium of the body.

Enzymes and transport molecules have been reported to produce F.R.s as an ordinary outcome of their catalytic functions and the auto-oxidation reactions of organisms are said to make F.R.s from the spontaneous oxidation of biological molecules in non-enzymatic electron transfer.

There are biological processes that usually result in the creation of F.R.s under standard conditions that the body is able to utilise in some instances while the body is able to regulate their presence but F.R.s are normally lethal molecules which are insistently created, where they uncontrollably attack and harm molecules inside the cells.

The harm caused by F.R.s is frequently assessed as products of DNA breakage or fragmentation, peroxidised lipid products and protein carbonyls. As a whole the process of damage that is caused by F.R.s to cells is known as oxidative stress.

Free radicals are normally produced by bonds binding the elements splitting in a manner that result in a molecule or molecules with an odd number of unpaired electrons but once a weak bond splits it leads to the formation of F.R.s (El-Aal, 2012)..

Free radicals are incredibly unstable compounds that react speedily with other molecules that are nearby the F.R. because it's attempting to capture an electron from the nearby molecule to gain stability.

PUFA's perform as exceptional substrates for L.P., because of the existence of bis-allylic methylene groups that are found throughout the molecules, the carbon-hydrogen bonds on these activated methylene units have lower bond dissociation energies thus rendering the hydrogen atoms more readily susceptible to being abstracted in radical reactions.

Lipids damaged due to excessive oxidative usually result in the formation of lipid hydroperoxides which are non-radical intermediates from cholesterol, glycolipids, cholesterol esters, oxidised unsaturated fatty acids and phospholipids.

The homolytic fission of water produces the hydroxyl radical, which is a very reactive ROS species and the combustion of hydrocarbons at high temperatures leading to the breaking of carbon-carbon, carbon-hydrogen and carbon-oxygen bonds is a F.R. process (El-Aal, 2012).

1.6 Problem statement

With the ever increasing rates of incidence by diseases known to cause oxidative stress, it is to natural products, in the form of plant material for the project, were we turn to provide sources of new compounds that can be elucidated and used to combat these diseases.

Anti-oxidants are found in abundance in nature but there are few synthetic alternatives to these natural anti-oxidants. Not many plants from the Eastern Cape have their anti-oxidant activity documented though it is documented that many possess healing properties and may contain undiscovered anti-oxidants.

With the increase in the pace in lifestyle of today's modern human being, fast food and fast food outlets have become popular as people don't have the time to cook a full meal and continuous frying of oils can lead to the oil undergoing chemical reactions such as polymerization, oxidation and hydrolysis due to cost cutting measures like repeatedly frying same batch of oil to increase profits and reduce wastage.

This continued heating leads to the oil having a multitude of oxidative products like aldehydes and hydroperoxide that are engrossed into the fried food (Ali Hassan & Abd El-Aal, 2012).

1.6.1 Justification

Hippobromus pauciflorus plant extracts shown to contain antibacterial, anti-fungal and anti-inflammatory properties, research on plant extracts have shown that the plant possesses secondary metabolites for example steroids, flavonoids, tannins, terpenes, cardiac glycosides and saponins that are known to be involved in providing above mentioned properties and there is little scientific literature on its anti-oxidant properties and activity.

Little is known about the *Pentanisia* genus in general and much more in depth research can be conducted in terms of its biological properties and compounds that can be isolated and elucidated from the genus and *pentanisia prunelloides* specifically.

Rubiaceae family is known to possess alkaloids, flavonoids, terpenoids, amino acids and carbohydrates, secondary metabolites that are known to possess anti-bacterial and antimicrobial properties are also found in this plant family.

1.7 Aim and Objectives

1.7.1 Aim

To identify and isolate compound(s) from the *Huppobromus pauciflorus* and *Pentanisia prunelloides* by means of thin layer chromatography and Column chromatography and to assess their biological properties.

1.7.2 Objectives

- To extract secondary metabolites by means of solvent extraction.
- To identify compounds present in the extracts using thin layer chromatography.
- To elucidate a compound or compounds by means of column chromatography.
- To quantify to polyphenols and flavonoids present in the extracts
- To assess the anti-oxidant properties of the plants by using FRAP and DPPH assays
- To determine whether the extracts inhibit lipid peroxidation.

1.8 Research Questions

Does one or both plant sample contain a compound can elucidated by means of chromatography and spectral analysis?

Will the extracts be able to be used to quantify phenols and/or flavonoids that may be present?

Will the extracts be able to demonstrate anti-oxidant activity of one or both plants?

Will the extract of one or both plant samples be able to inhibit lipid peroxidation?

1.9 Hypothesis

That from *Huppobromus pauciflorus* and/or *Pentanisia prunelloides* there is one or more compounds that can be elucidated. That the extracts will show that one or both plant samples possess the biological activities being investigated.

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Chapter 2

2.1 Secondary metabolites

Secondary metabolites are defined as organic molecules that do not participate in the usual development and growth of an organism but they play a vital element in the defence and survival of plants. They are being bioactive compounds that are found in a myriad of medicinal plants, colourants, food and spice plants.

They are reported to be generated at their highest levels during the plants' transition from its active growth phase to its stationary phase. Secondary metabolites are composed of an exceptionally varied group of natural products that are generated by an array of organisms i.e. animals, plants, algae and fungi.

Most can be easily classified according their biosynthetic origin but the compounds that tend to be tricky to classify are usually related to a confined set of species within a phylogenetic group (Agostini-Costa, Vieira, Bizzo, Silveira & Gimenes, 2012).

2.2 Essential Oils

Essentail oils are natural and volatile compounds that are found throughout the plant (roots, leaves bark, stems, etc.) and possess an array of pharmacological effects for instance anti-fungal, anti-viral and antibacterial properties to list a few.

They are a complex mixture of volatile compounds made up of monoterpenes, sesquiterpenes and their oxygenated compounds and are characterised by their often pleasant fragrance while also providing protection to the plant against predators and disease.

They are fat soluble but fatty lipids or fatty acids from vegetables and animal oils are not considered to be essential oils. Single or combined extracts of essential oils can be used to excite the olfactory nerve which transmits messages to the brains limbic system that trigger physiological responses, hence the use of essential oils in aromatherapy. They are used as preservatives, flavouring agents and as fragrances in the cosmetics industry.

Volatile essential oils can be extracted using steam distillation though hydrodistillation which is a frequently used extraction method and are qualitatively and quantitatively measured by use of Gas Chromatography-Mass Spectroscopy (GC-MS) and Gas Chromatography (GC). Examples of essential oils are limonene, *cis*-tagnose, elixene, spathulenol, sabinene, terpin-4-ol and zierone (Kabera, Semana , Mussa & He, 2014; Mlala, 2016).

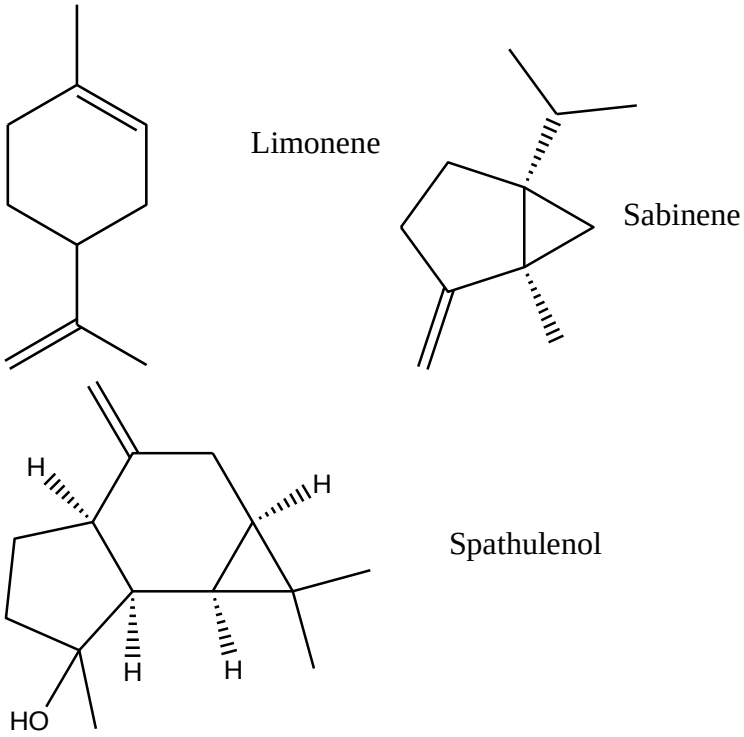


Figure 2.1: Examples of Essential oils

2.3 Hydrocarbons

They are the least polar of organic natural product and normally contain an odd number of carbon atoms. Hydrocarbons do not have any heteroatoms and they may be saturated or unsaturated, examples of hydrocarbons are n-heptane, n-nonacosane and n-hentriacontane.

2.3.1 Saturated hydrocarbons

Are hydrocarbons that are normally found as the waxy coatings on leaves. Turpentines, which are usually utilised as paint removers, are made up of simple hydrocarbons for instance n-heptane as it has been predominantly located in conifers.

2.3.2 Unsaturated hydrocarbons

Ethylene is the simplest example of an unsaturated hydrocarbon. Unsaturated hydrocarbons that are large are usually common as plant waxes in plants and with an increase in the degree of unsaturation and chain length within the hydrocarbon, the waxier the hydrocarbon becomes (Brielmann, 1999).

2.4 Terpenes

These compounds are amongst the most prevalent and chemically diverse sets of secondary metabolites found; they are a distinct set of hydrocarbon-based compounds whose shape might be derived from isoprene leading to shapes which may be divided into isopentane.

To synthetically manufacture monoterpenes, Isopentenyl pyrophosphate and dimethyl allyl pyrophosphate are reacted to generate geranyl pyrophosphate leading to monoterpenes. The reaction of farnesyl pyrophosphate leads to sesquiterpenes.

Triterpenes are created by two equivalents of farnesyl pyrophosphate reacting with each other. They are categorised by the amount of five-carbon atom units that the terpene contains (Brielmann, 1999).

2.4.1 Hemiterpenes (C5)

They are the simplest example of several five-carbon compounds which contain an isopentane skeleton. Isoamyl alcohol, isovaleric acid, seneciocic acid, tiglic acid, angelic acid and β-furoic acid are a few examples of these compounds (Brielmann, 1999).

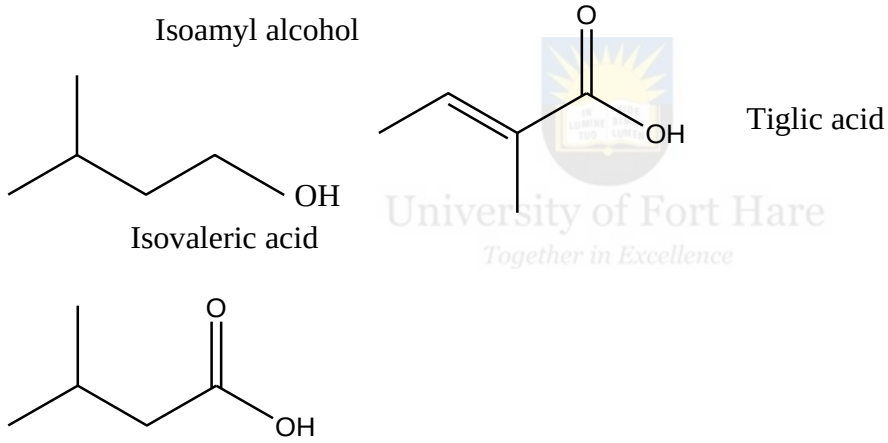


Figure 2.2: Examples of Hemiterpenes

2.4.2 Monoterpenes (C10)

Often are the main parts of numerous essential oils and have financial significance and value as colourants, flavourants and perfumes. Common aliphatic examples of monoterpenes encompass myrcene, geraniol, linalool and open structured monoterpenes examples include camphor, menthol, pinene and limonene (Brielmann, 1999).

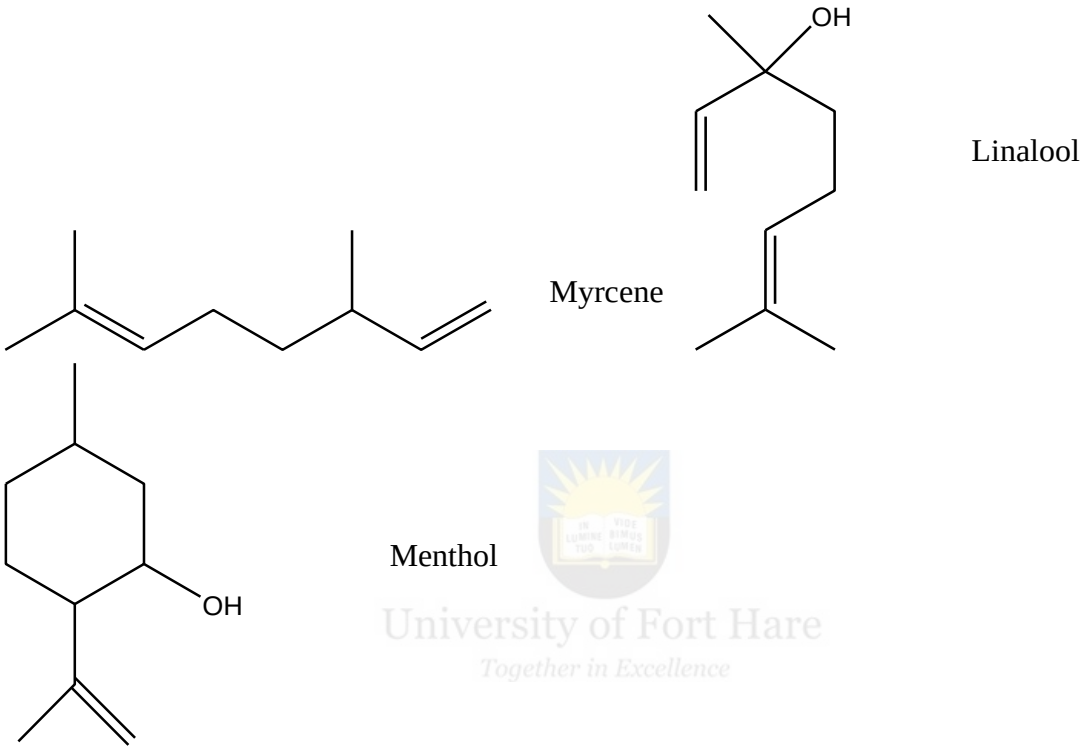


Figure 2.3: Examples of Monoterpenes

2.4.3 Sesquiterpenes (C15)

Sesquiterpenes are consequentially derived from three isoprene units linked to form sesquiterpenes and occur in various forms: Linear, bicyclic and tricyclic frameworks. Sesquiterpenes are generally considered to be essential oils and examples include ώ-bisabolene, lanceol, perezone, humulene, α-cadinene, eudesmol, caryophyllene, abscisic acid, helenalin, psilostaychin and tetrahydroridentin B (Brielmann, 1999).

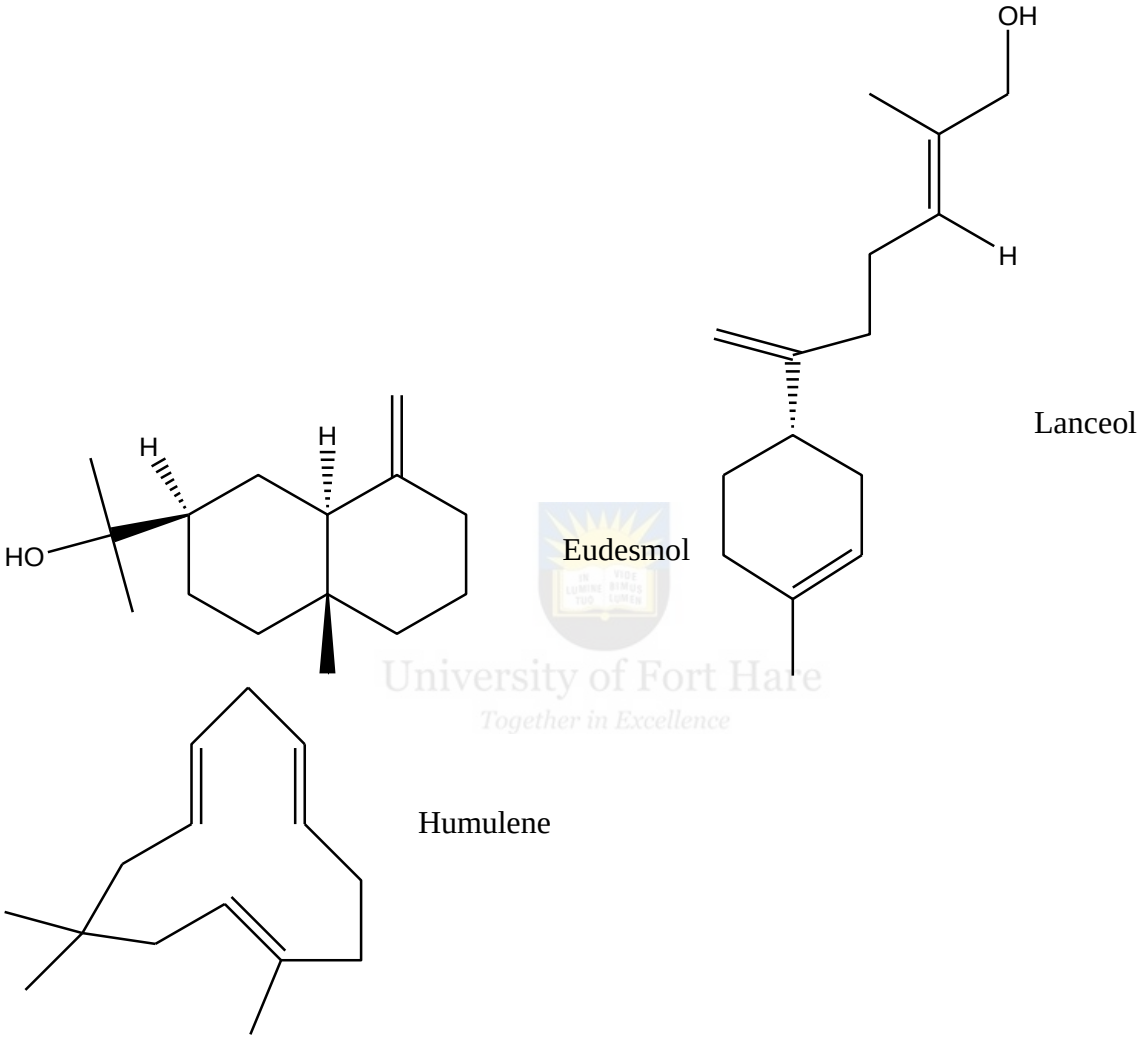
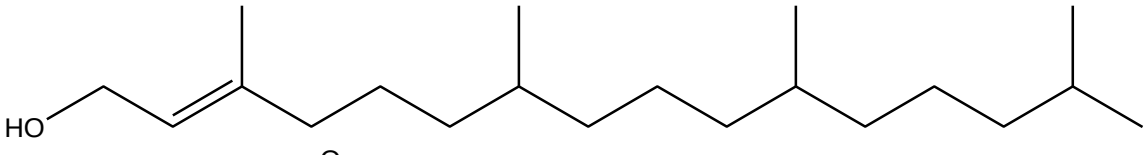


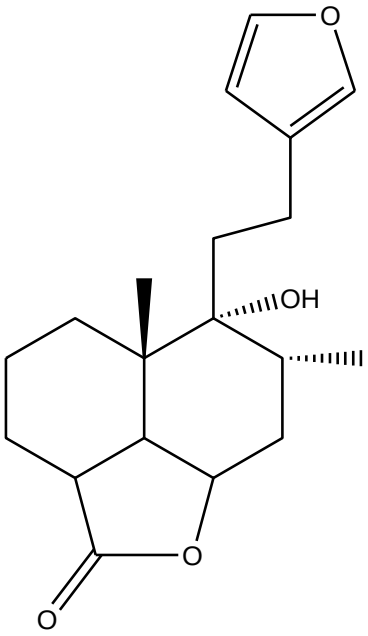
Figure 2.4: Examples of Sesquiterpenes

2.4.4 Diterpenes (C20)

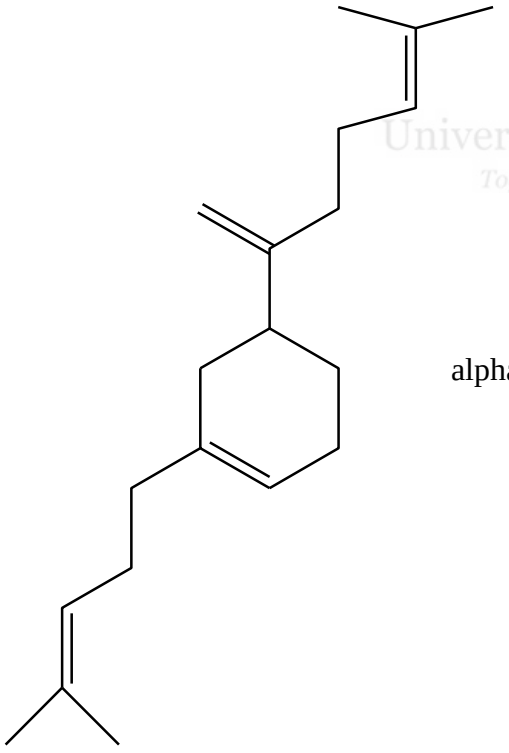
Diterpenes are a collection of compounds which are broadly varied based on isoprene groups they are not considered to be essential oils due to their higher boiling point but are instead considered as resins. They occur in various structural types in nature. Examples of diterpenes include α-camphorene, marrubin, phytol, zoapatanol, inumakilactone a, gibberelic acid and taxol (Brielmann, 1999).

Phytol





Marrubin

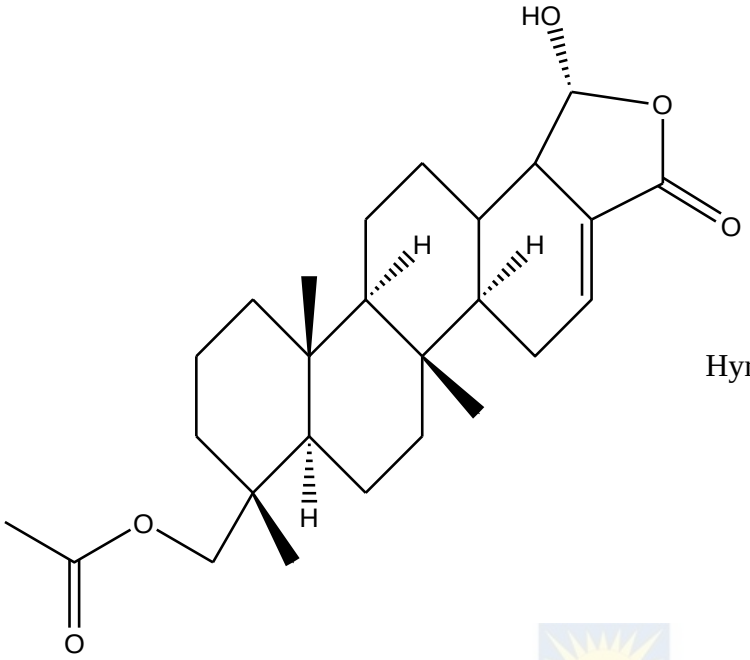


alpha-camphorene

Figure 2.5: Examples of Diterpenes

2.4.5 Sesteterpene (C25)

Sesteterpene are reported to be very rare (Brielmann, 1999).



Hyrtios sesterterpene

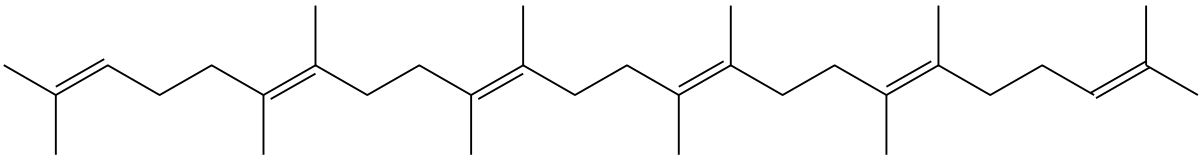
Figure 2.6: Example of a Sesteterpene

2.4.6 Triterpene (C30)

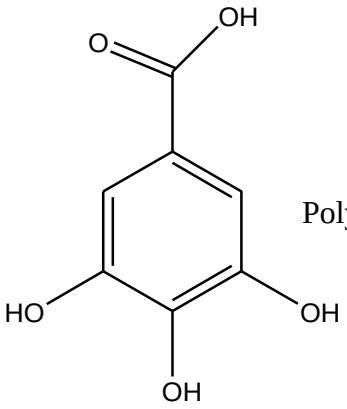
Triterpenes have their basis from six isoprene monomers linked together and Squalene is utilised to biosynthetically synthesise them. Frequently they occur as colourless solids with high melting points. There are some significant sets of triterpenes: common triterpenes, cardiac glycosides, steroids, sterolins and saponins (Brielmann, 1999).

2.4.6.1 Common triterpenes

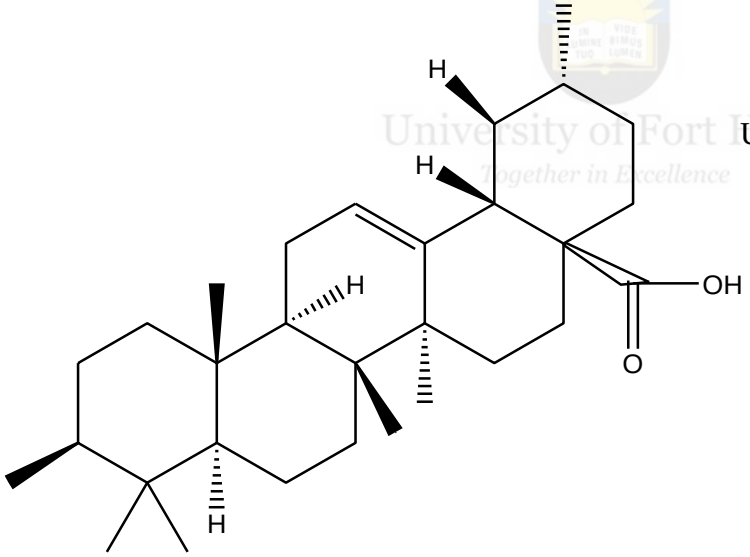
Only a few are widely distributed among the families of plants with common examples of these triterpenes including ursolic acid, oleanic acid, limonins, squalene, polygallic acid, azadirachtin and α-amyrin (Brielmann, 1999).



Squalene



Polygallic acid



Ursolic acid

Figure 2.7: Examples of Common Triterpenes

2.4.6.2 Sterols

Sterols are typically steroid in structure with nearly all plant steroids being hydroxylated at carbon number 3. In animals, steroids have a deep significance as they are pro-vitamins, coenzymes and hormones which are vital in the maintenance of animal health. Examples of sterols include cholesterol, fucosterol, ergosterol, and stigmasterol (Brielmann, 1999).

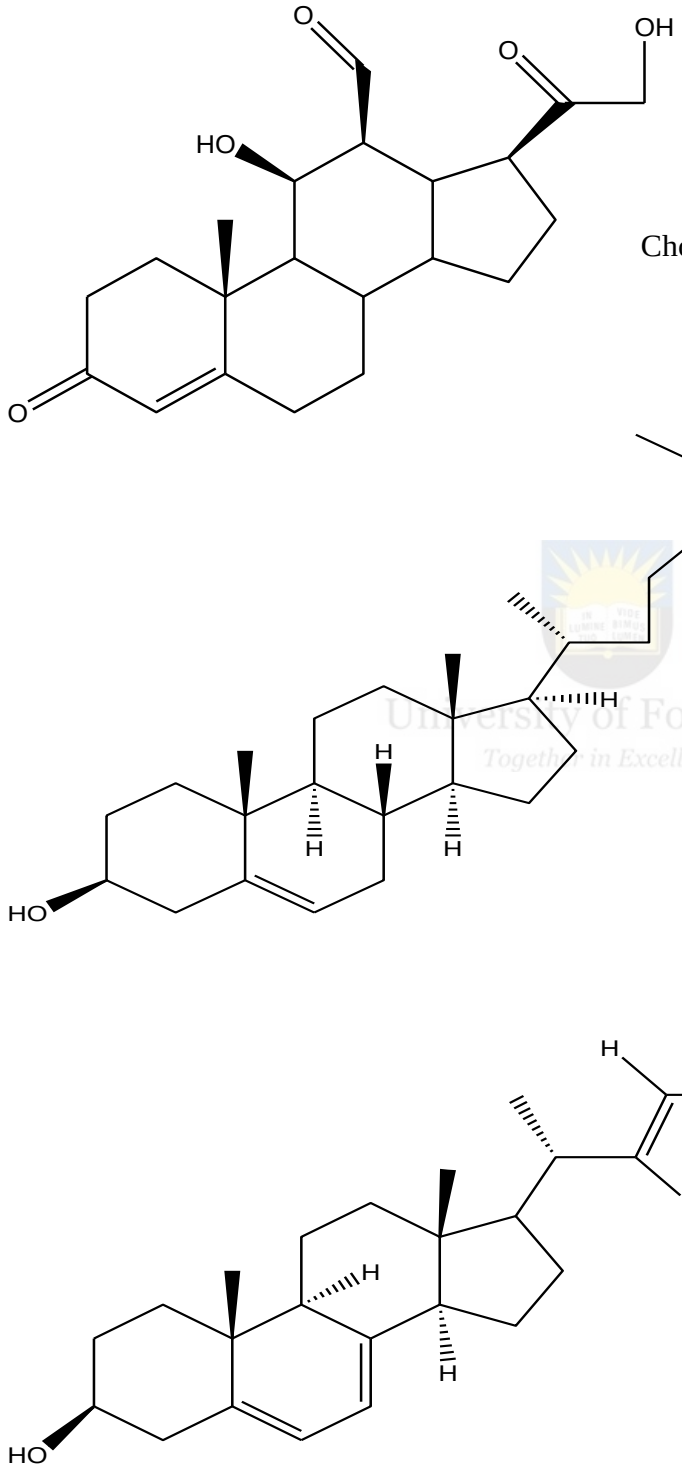


Figure 2.8: Examples of Sterols

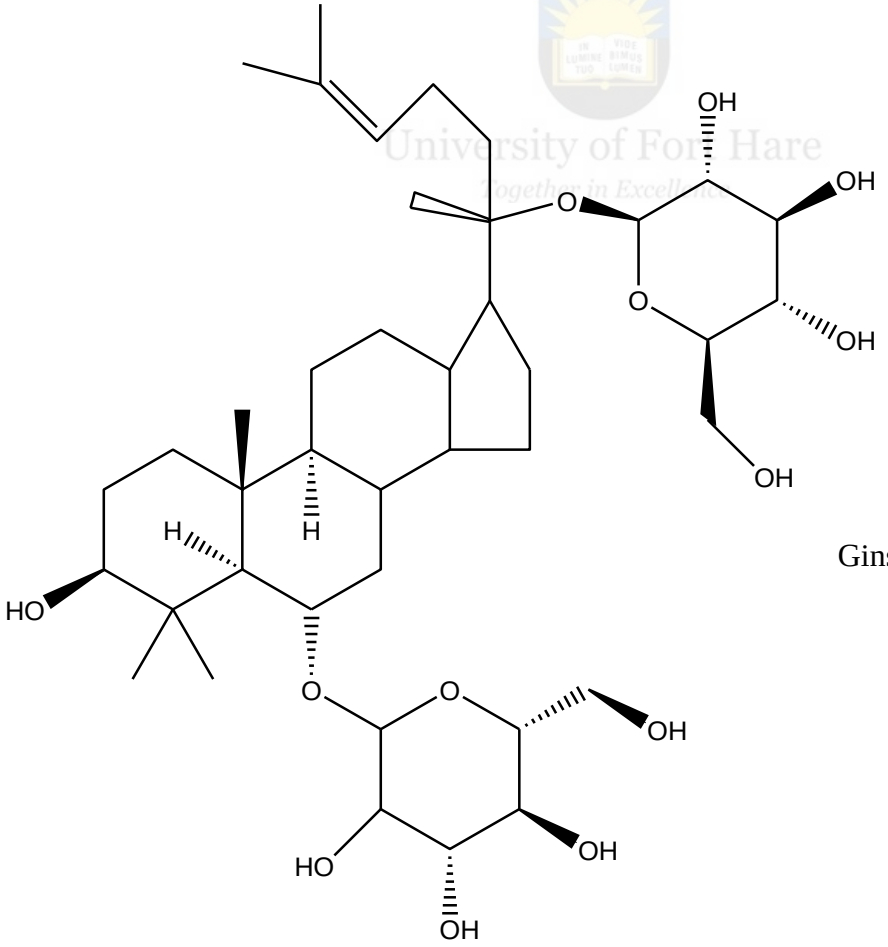
2.4.6.3 Saponins

Are triterpene glycosides which possess a high-molecular-weight that have a sugar bound either to a triterpene or a sterol. Saponins are found abundantly in plants and occur as glycosides that are made up of two parts namely glycone (a sugar) and aglycone or genin (a triterpene).

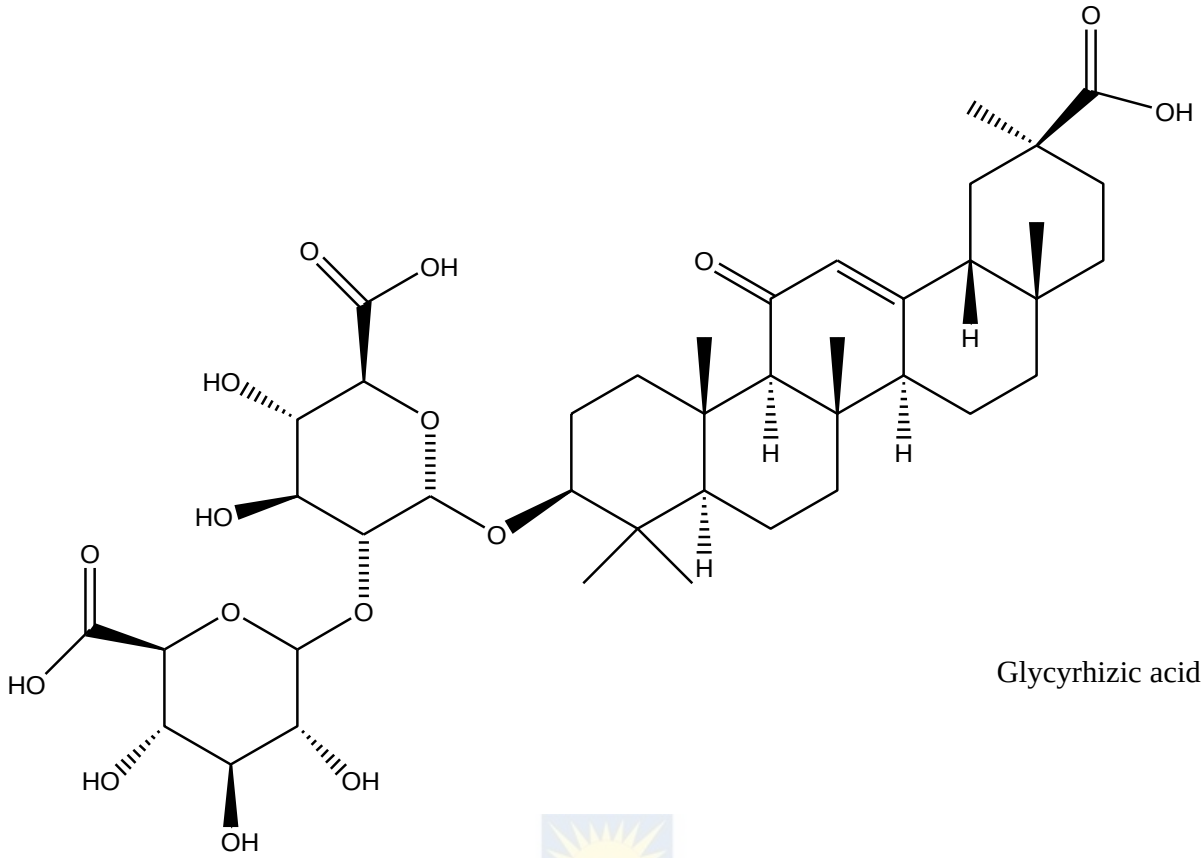
Saponins are compounds that contain an active portion that form colloidal solutions in water, exhibit piscicidal properties, have a bitter taste, they also form foam readily in water and possess detergent properties due to the blend of lipophilic sugars at the end of the compound which enables them to lessen the surface tension, causing the detergent property or soap-like effect on membranes and skin.

Many plants containing saponins have been traditionally used as soaps; are ingredients of many plant drugs and folk medicines. They exhibit many biological- (anti-microbial, anti-oxidant, ichthyocide, haemolytic and molluscacide) and physical properties (solubilisation, foaming, emulsification, bitterness and sweetness).

They are either polydesmodic or monodesmodic subject to the amount of sugar moieties that are attached. They have been used in a number of ways in the pharmaceutical industries, cosmetics and food as well as in soil bioremediation. Classes of saponins inclue triterpene, steroids, steroid alkaloid, monodesmodic and polydesmodic. examples ginsenoside and glycyrrhizinic acid (Brielmann, 1999).



Ginsenoside



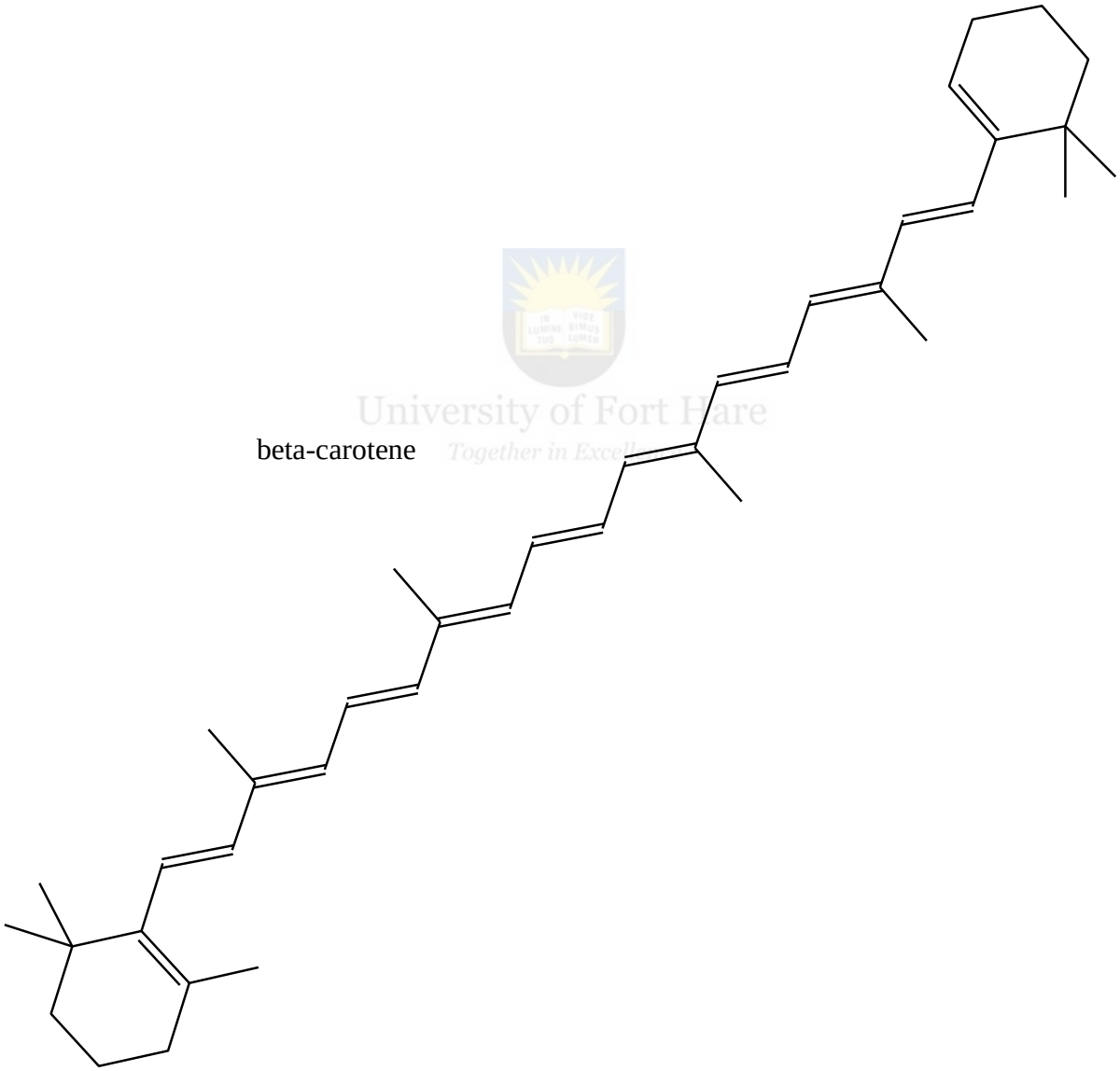
Glycyrhizic acid

Figure 2.9: Examples of Saponins

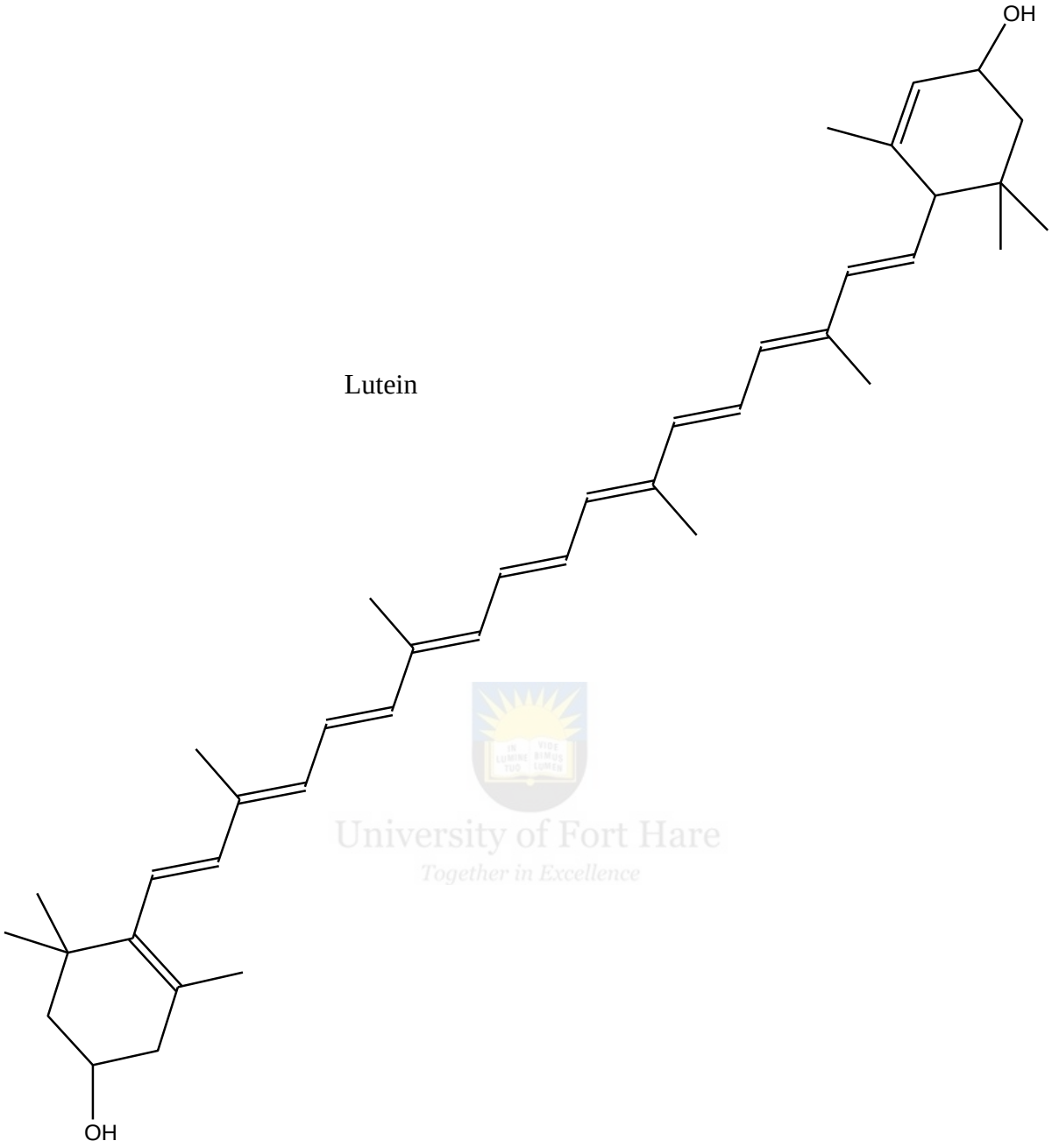
2.4.6.4 Tetraterpene (C40)

More than 650 tetraterpenes or sometimes referred to as carotenoids have been isolated (Agostini-Costa et al, 2012). Most common examples are carotenoids which are biosynthetically synthesise from lycopene. Cyclisation at one end of a carotenoid yields ϒ-carotene while cyclisation of both ends of the carotenoids yields β-carotene.

Tetraterpenes account for the brightly coloured pigments in plants because of their polyene structure and numerous double bonds they possess. Isomers are possible for basic tetraterpenes and are believed to provide protection to plants from over-oxidation that is catalysed by light absorbing pigments like chlorophylls. Examples include Lycopene, α-carotene, β-carotene, Lutein and Rhodoxanthin (Brielmann, 1999).



beta-carotene



Lutein

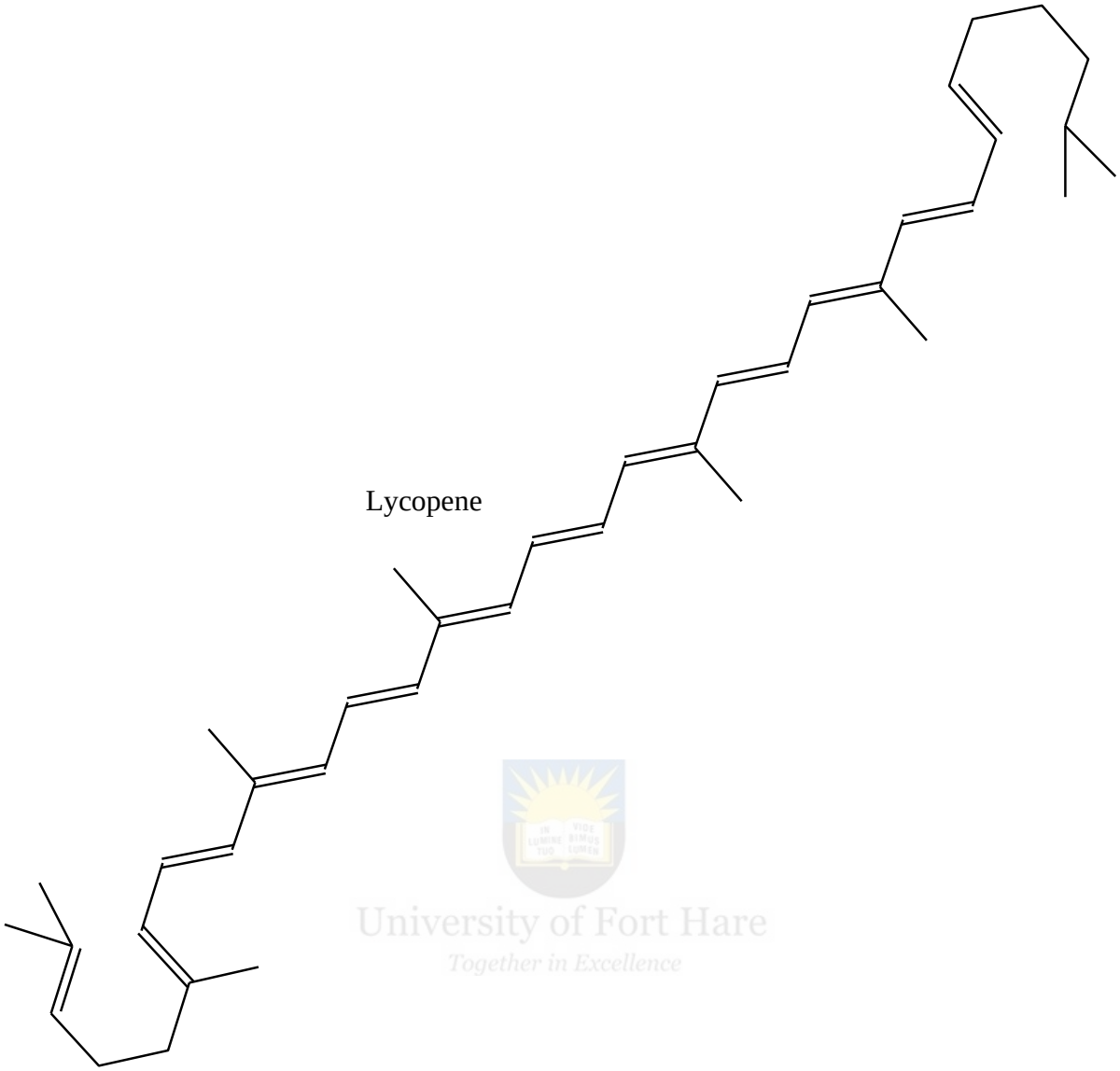


Figure 2.10: Examples of Tetraterpenes.

2.4.7 Flavonoids

Flavonoids are part of the group of polyphenols which are water soluble pigments that are located in the plant cell vacuoles and contain two benzene rings that are separated by a propane unit and are derived from Flavone.

Flavonoids are widely distributed in plants and the more conjugated flavonoids are often brightly coloured and responsible for flower colouration producing red, blue or yellow pigments in petals which aids in attracting pollinators to ensure plant species survival.

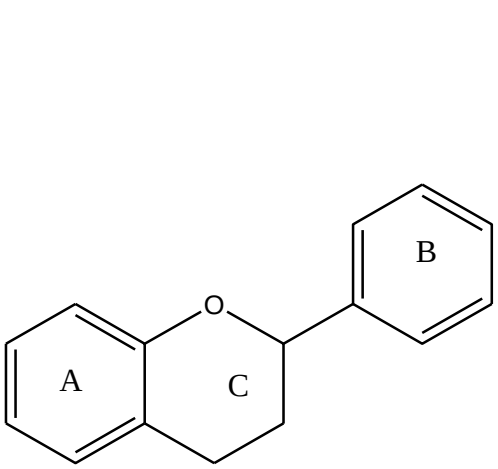
Flavonoids are usually discovered in plants in their glycoside form that may lead to complications in structure classifications and determinations.

In higher plants, they are reported to take part in symbolic nitrogen-fixation and UV filtration. Also they perform as physiological regulators cell cycle inhibitors, and as well as chemical messengers.

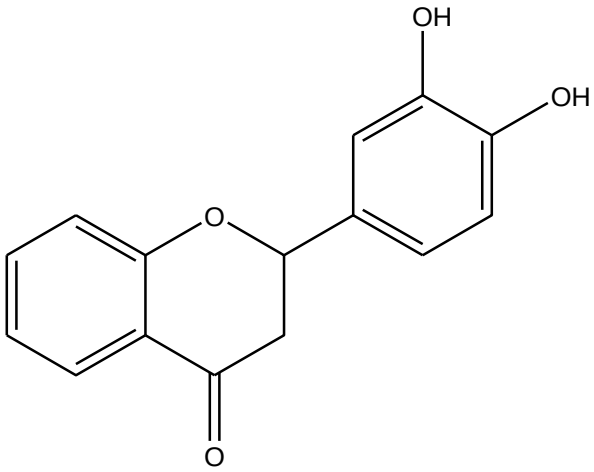
These properties that flavonoids are reported to exhibit are some of the many reasons why flavonoids are popular and of a lot of interest. Flavonoids have anti-cancer, anti-oxidant, anti-viral, anti-inflammatory and anti-allergic properties.

There are distinct classes of flavonoids that are notable by additional oxygen-containing heterocyclic rings and hydroxyl groups.

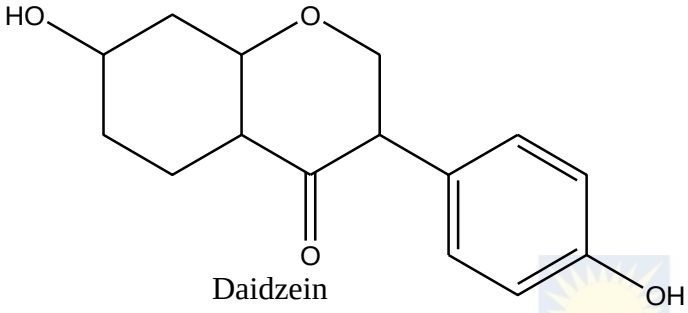
Classes encompass: catechins, flavonols, xanthones, flavones, leucoanthocyanidins, flavanonorels, aurones, chalcones, condensed tannin, isoflavones and anthocyanidins (Brielmann, 1999).



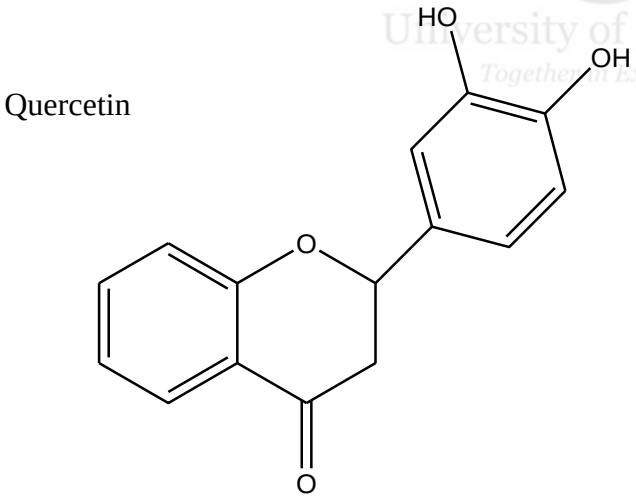
Basicstructure



Luteolin (Flavone)



Daidzein



Quercetin

Figure 2.11: Examples of Flavonoids and the basic structure of Flavonoids

2.4.8 Tannins

Tannins are compounds are found commonly in vascular plants occurring within the woody tissues. Tannins contain phenolic compounds that react with proteins to form water-insoluble copolymers.

Tannins are said to be either condensed or hydrolysable and have their name originating from the French word “Tanin” meaning tanning substance while they also encompass a broad assortment of natural polyphenolics.

They consist of an extremely varied collection of polymers and oligomers that are water soluble besides some structures that have a high molecular weight and may create complexes with cellulose, minerals and starch as well.

Condensed tannins are biosynthetically created by the condensation of catechins to form polymeric networks. Hydrolysable tannins are created from gallic acid. Tannins can be synthesised via the shikimic acid pathway (i.e. the phenylpropanoid pathway) and the same pathway can be used in the formation of other phenolic compounds like isoflavones, aromatic amino acids, coumarins and lignins.

According to literature, plants that contain tannins have been used against diarrhoea, a diuretic in treating duodenal and stomach tumours whilst also being utilised as an anti-inflammatory agent. Examples of tannins include gallotannins and eligotannins (Brielmann, 1999; Agostini-Costa et al, 2012).

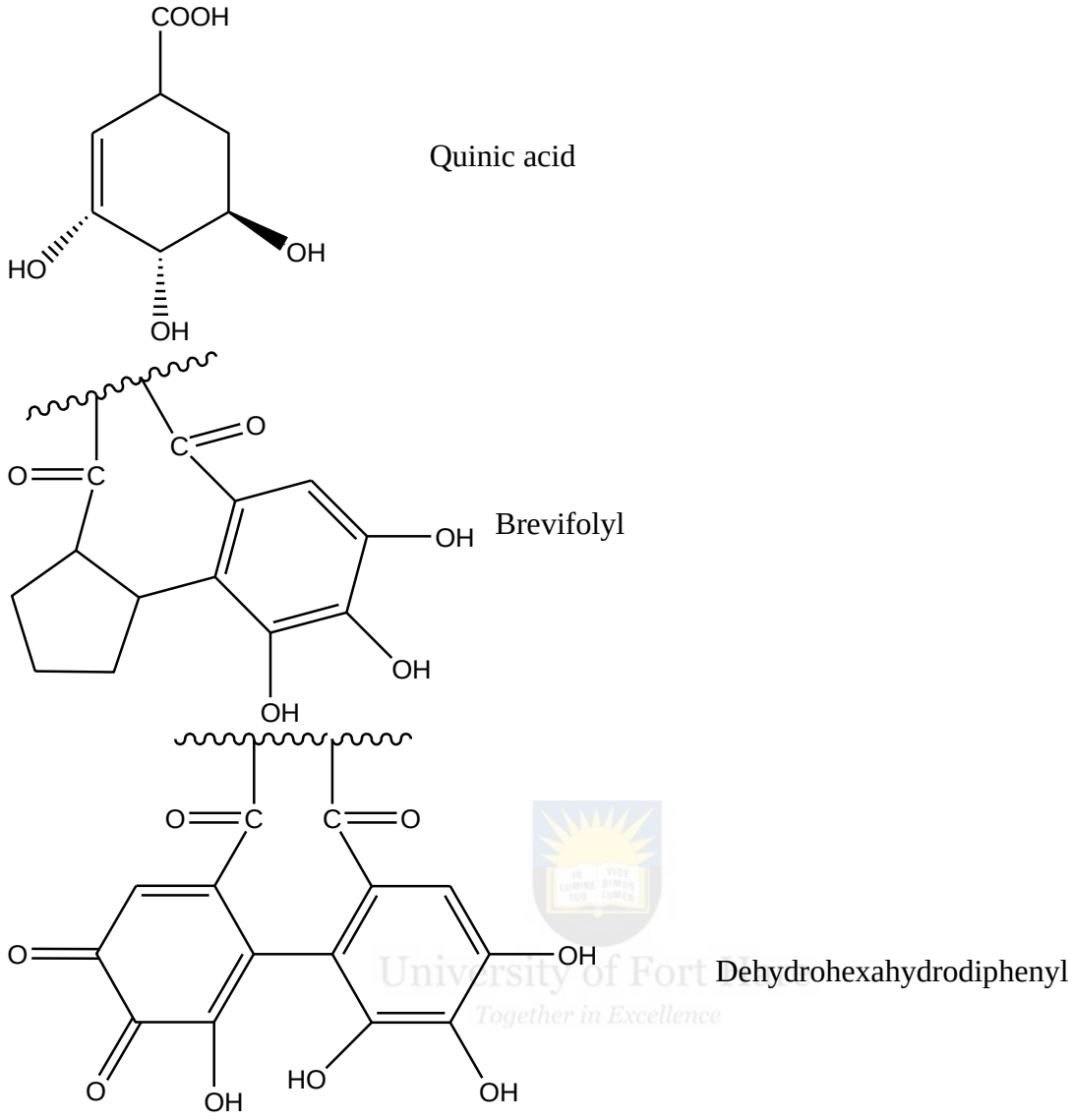


Figure 2.12: Examples of Tannins

2.4.9 Alkaloids

Alkaloids are defined as basic compounds that are synthesised by living organisms that contain one or more heterocyclic nitrogen atoms. Alkaloids are created by various organisms like bacteria, animals and fungi but mainly by plants and in addition to carbone nitrogen and hydrogen; they can as well have oxygen, bromine, sulfur, phosphorus and chlorine.

With the exception of a few compounds, they are mostly soluble in hydroethanolic media and typically exist in nature as salts like sulfates or chlorides or in plants as nitrogen oxides.

Plenty are created from amino acids but they can also be obtained from the modification of various molecules including terpenes, steroids and polyphenols and many alkaloid names tend to end with the suffiexes –in and -ine.

Alkaloids are abundant among plants and make up a large number of secondary metabolites with more than 12000 alkaloids having been isolated and documented. They are well known for their potent and varied pharmacological properties including being an insecticide, anti-fungal, anti-bacterial, antimitotic and anti-tumour to name a few, for all the beneficial properties there some alkaloids that have toxic and potentially fatal properties.

Many common drugs are alkaloid based. When treated with an acid, alkaloids forms water soluble salt. Biosynthetically, they can be derived from terpenes, aromatics or amino acids subject to the specific alkaloid structure that is wanted. Because of their abundance and assortment alkaloids are usually obtained from natural sources rather than being synthesised in the laboratory.

There are many different ways to classify this group of compound into specific groups due to their diverse structures but one system that is used is based mainly on either the type of ring-structure or the botanical taxa in which the alkaloids are found and they go as follows:

Amaryllidaceae alkaloids, Piperidine alkaloids, Betalanin alkaloids, Methylxanthines, Imidazole alkaloids, Phenethylamines, Indole alkaloids, Steriodal alkaloids, Pyrrolidine alkaloids, Diterpenoid alkaloids, Monoterpenoid alkaloids, Peptide alkaloids, Pyridine alkaloids, Isoquinoline alkaloids, Tropane alkaloids and Quinolizidine alkaloids.

Examples include Chelidonine, Lycodopine, Senicionine, intermedine, caffeine, hygrine, cocaine, Berberine, morphine, codeine and Ajmaline to name a few (Brielmann, 1999; Agostini-Costa et al, 2012).

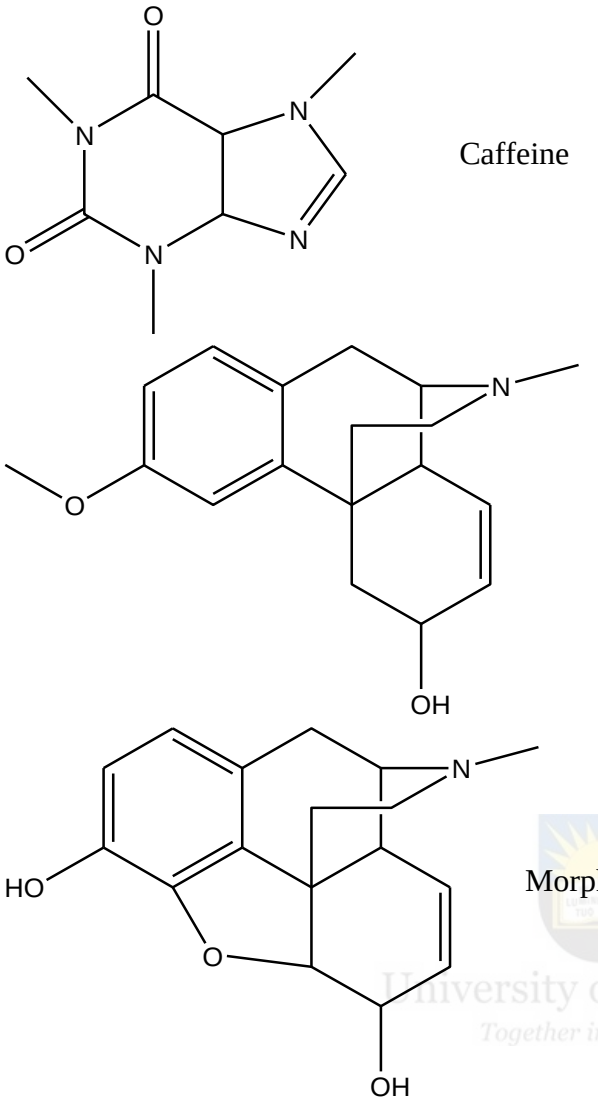


Figure 2.13: Examples of Alkaloids

2.4.10 Glycosides

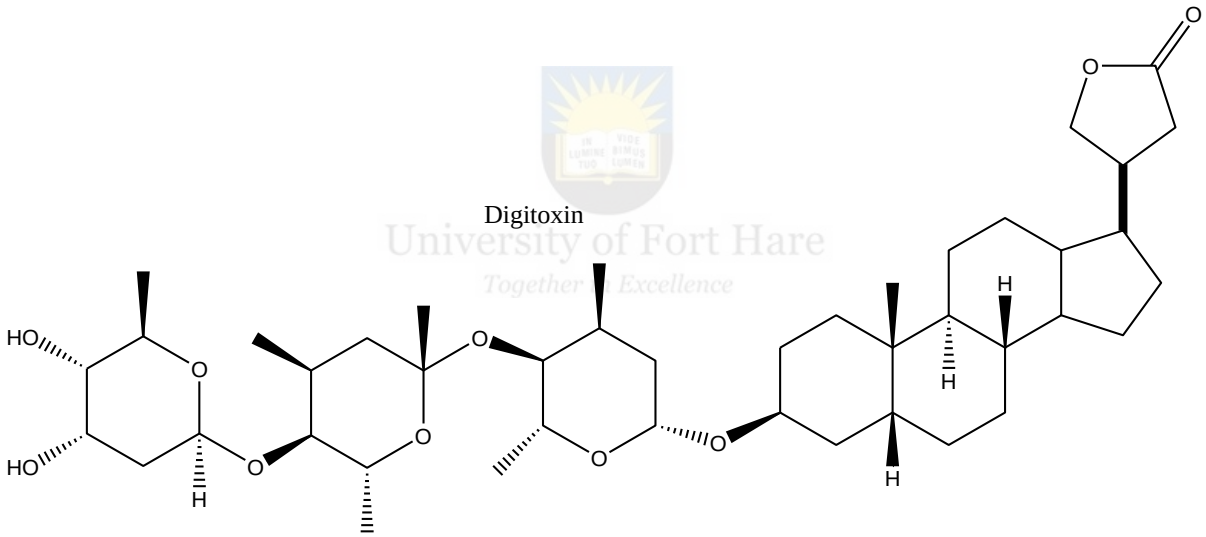
Cardiac glycosides are a big group of natural compounds primarily containing core structures which have a steroid nucleus with a cardenolides (five-membered lactone ring) or a bufadienolides (six-membered lactone ring) and sugar moieties.

They are compounds that are heterogeneous in structure and are characterised by a sugar moiety or a portion that is attached by glycosidic bonds to one or more non-sugar portion (usually a triterpene) and may be alcoholic, sulfur or phenolic compounds.

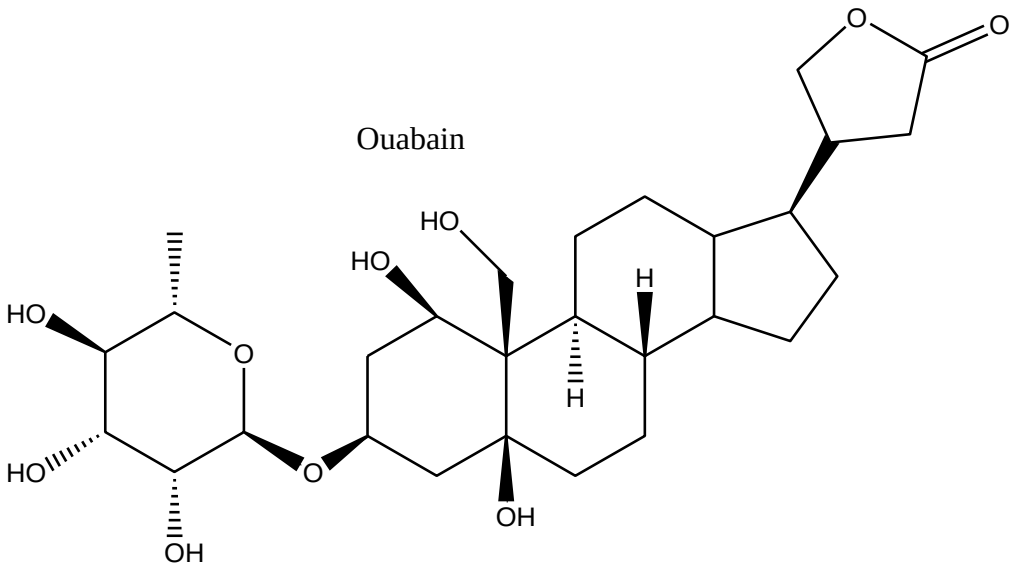
Numerous plants amass chemicals as inactive glycosides that are activated using enzyme hydrolysis and thus they are classed as pro-drugs since they are inactive until they are hydrolysed by enzymes resulting in the release of aglycone.

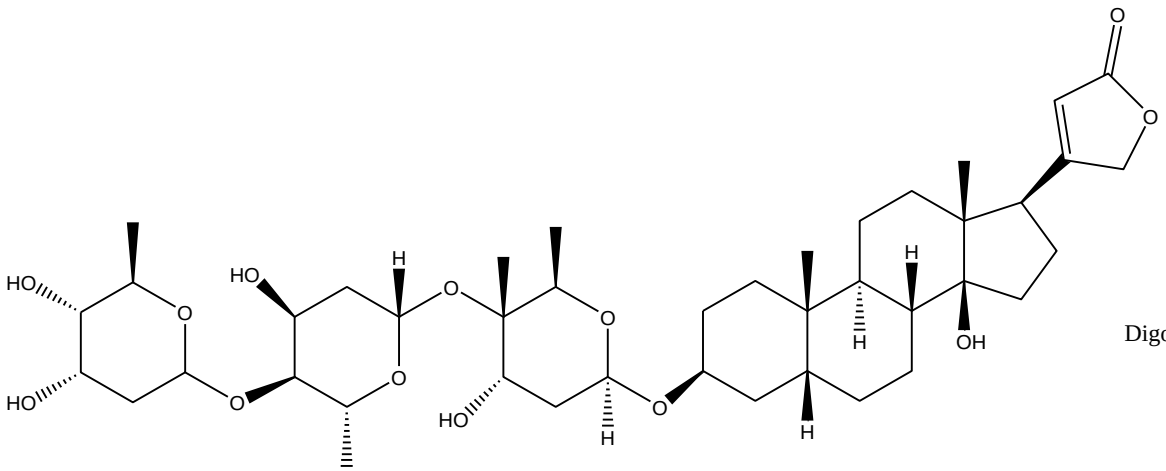
Classification of glycosides is based on the type of aglycone which comes in a large range of molecule type. It is reported that glycosides have exhibited expectorant, digestive, anti-cancer and sedative properties. Examples include digoxin, digitoxin, ouabain, and oleandrin (Pongrakhananon, 2013).

Digitoxin



Ouabain





Digoxin

Figure 2.14: Examples of Glycosides

2.5 Extraction

2.5.1 Solvent extraction

Solvent extraction is based on the principle of solid-liquid solvent extraction, that is when a solid matter is immersed into a liquid solvent, all the constituents that are soluble in that solvent migrate from the solid matter towards the liquid solvent.

Thus, the extraction by using a solvent of a plant leads to a mass transfer of suitable active material to the solvent and occurs across a concentration gradient. The mass transfer of active material from the plant into the solvent continues till it reaches equilibrium i.e. when the concentrations of soluble active materials and solvent are equal and when equilibrium is reached, there will no longer be the transfer of active material, from solid matter to the solvent (Mlala, 2016).

2.6 Analytical Techniques

2.6.1 Ultraviolet/Visible (UV/Vis) spectroscopy

2.6.1.1 Spectroscopy

Spectroscopy is defined as the quantifiable assessment of the properties of reflection or transmission of an analyte and encompasses the usage of a spectrophotometer, which is a device that used to assess the properties of reflection or transmission of the material intensity as a function of wavelength from the light source.

UV/Vis spectroscopy is utilised to study the changes of electronic energy levels within the molecules of the analyte due to transfer of electrons from a lower energy orbital to a higher energy orbital.

UV/Vis spectroscopy has its basis on the scattering, absorption, refraction, reflection and diffraction properties of a substance (Roberts, Power, Chapman, Chandra & Cozzolino, 2018).

As a result of the absorption of visible or ultraviolet radiation by an analyte, there are transitions between the electronic energy levels of the analyte, UV/Vis spectroscopy is likewise known as electronic spectroscopy and the data obtained from this spectroscopy when it is used in combination with information provided by nuclear magnetic resonance and infrared spectra leads to valuable structural elucidations. (Kumar, 2006)

2.6.1.2 Spectrophotometer

A spectrophotometer is a device that is frequently utilised for the assessment of the transmittance or reflectance of solutions and translucent or murky solids.

Traditionally they utilise a monochromator that has a diffraction grating to created analytical spectrum needed for analysis while the majority of contemporary mid-infrared spectrophotometers utilise a Fourier transform method to get the spectral data of unknown analytes (Bosch Ojeda & Sánchez Rojas, 2009; Cioabla, 2013).

Important constituents of a spectrophotometer are the light source that produces an electromagnetic radiation band. Detectors, there could be one or more, to record the intensity of light radiation and a dispersion device that chooses the desired radiation band of a particular waveband and a sample area (Bosch Ojeda & Sánchez Rojas, 2009; Cioabla, 2013).

Various scientific fields like biochemistry, chemistry, physics, material science, food technology and molecular biology involve the usage of spectrophotometers. There are two types of spectrophotometers that are commonly used: a double-beam and a single-beam spectrophotometer.

A single-beam spectrophotometer assesses the light intensity of a radiation band before and after as analyte is implanted while a double-beam spectrophotometer evaluates light intensities amid the two light paths, where one of those paths has the reference sample and the other the analyte.

UV/Vis spectrophotometers have applications in detecting halogens and halogenated compounds, detecting aromatic compounds, colour measurement as well as detecting sulfur compounds, inorganic compounds, pharmaceutical compounds, fermentation processes monitoring and analysis of gaseous pollutants in air (Bosch Ojeda & Sánchez Rojas, 2009; Cioabla, 2013).

These advantages of using a spectrophotometer are that a shutter from the light source of the spectrophotometer panels the quantity of light coming from the specialized lamp that permits through the analyte whilst also being the only shifting part of a UV/Vis spectrometer and the simplistic design of the instrument is also advantageous in terms of low maintenance.

The time taken to assess the analyte in UV/Vis spectroscopy is an incredibly rapid process, concentrations of the analytes do not have to be high and UV/Vis spectroscopy is non-destructive procedure to an analyte and is highly sensitive in detecting organic compounds.

Whilst the disadvantage are that there is no solitary lamp that produces all of the wavelengths that are needed to analyse the analytes, UV/Vis spectrometers need to be frequently calibrated in order to remain the accurate and precise and stray light can cause problems for UV/Vis spectrometers (Cioabla, 2013).

2.6.1.3 Light sources

There are two sources of light that are frequently utilised in UV/Vis spectrophotometers; one source is a deuterium arc lamp that generates a decent light intensity continuum over the ultraviolet area whist providing a handy light intensity continuum in the visible area.

The other light source is a Tungsten-halogen lamp that generates a decent light intensity over some parts of the ultraviolet region and the entire visible range.

A majority of spectrophotometers that are utilised to record the UV/Vis regions have both types of lamps in them. Therefore, there is either a source selector that is utilised to switch among the two lamps to select the fitting lamp or the light emitted by both light sources is combined to form one broadband light source (Kumar, 2006).

2.6.1.4 Dispersion devices

These devices instigate different light wavelengths to be spread at various angles when they are used in conjunction with an apt exit slit, dispersion devices that are utilised to pick out a definite light waveband from the light source. There are two types of dispersion devices: prisms or holographic prisms (Kumar, 2006).

When sunlight passes through a prism it produces a rainbow (light spectrum) from the sunlight, the same principle is used by prisms in spectrophotometers but are almost not used anymore in modern UV/Vis spectrophotometers.

Holographic prisms are produced from glass banks which are lined with extremely narrow grooves, this used to be mechanically done but with progresses in manufacturing techniques, now a holographic optical process is used to create these grooves.

The proportions of the grooves are in the same order as the wavelength of light that is to be spread with an Aluminium coating that is used to generate a source of reflection on the devices.

Light hitting the gratings is then reflected at different angles and they produce a wavelength that is dependent on a linear angular dispersion of light which have a similar wavelength and an advantage is that the angular dispersions are not sensitive to temperature.

They reflect light at various orders which overlap and the use of filters to make sure that only the desired light of reflection order contacts the detector. A monochromator consists of a dispersion device, an exit slit and an entrance slit (Kumar, 2006).

2.6.1.5 Detectors

They are used to change a light signal into an electrical signal to enable detection of the transmittance or reflectance of the analyte. Spectrophotometers usually have either a photodiode detector or a photomultiplier tube detector (Kumar, 2006).

A single photomultiplier is highly sensitive to low levels of light while photodiode detectors have a bigger active range and because they are solid-state devices, they are stronger than photomultiplier tube detectors.

In a photodiode detector, light that hits the semi-conductor material enables electrons to move within it thus draining the charge of a capacitor that runs throughout this material.
A diode array has a sequence of photodiode detectors positioned side by side on a silicon crys tal with a capacitor laid aside for each diode and is linked by a switch to an output line. The range of detections of photodiode detector is roughly around 170 – 1000 nm for silicon based detectors.

Conventional spectrophotometers work by having polychromatic light directed onto the entrance slit of the monochromator which then transmits a selected narrow light band light, which band flows across the sample area containing the analyte to the detector (Kumar, 2006).

With a single-beam instrument, a cuvette that is filled with the desired solvent is the positioned inside the spectrophotometer and then a blank recording is measured. The blank sample solution is then discarded and a sample solution is then poured into the same cuvette which is measured.

Photodiode array spectrophotometers work by having polychromatic light passing through the sample area which is then directed towards the polychromator's entrance slit and scatters the light towards a diode array on where every diode records a restricted bandwidth of the spectrum.

The light bandwidth that is sensed by a diode is relative to the dimensions of the polychromator's entrance slit and to the dimensions of the diode, while each diode is said to act an exit slit of a monochromator.

With a dual-beam or split-beam spectrophotometer, two cuvettes are required with both of them at first filled with a pure solvent so that a balance measurement is done. The measurement shows the difference in absorbance between the two light paths that are used in the spectrophotometer.

The sample cuvette is filled with the analyte solution for the assessment of the transmitted radiation and incident radiation. The resultant spectrum is corrected by subtracting from the balance spectrum (Kumar, 2006).

2.6.1.6 Measuring a spectrum

Molecular spectroscopy is defined as the research of the relations of electromagnetic matter and waves.

The degree of interaction of the sample with the radiation, whether transmittance or absorbance is determined by the measurements of both the intensity of the incident (radiation sans sample) and transmitted radiations (radiation with the sample).

Intensities are denoted I and I_o in the equation.

$$T = {}^{I}/_{Io}$$

$$\%\,T = {}^{I}/_{Io}\ x\ 100$$

$$A = -Log\ T$$

T =Transmittance, I = Transmitted radiation, I_o = Incident radiation, A = Absorbance

All the seemingly dissimilar types of electromagnetic radiations move at a similar velocity but are different from one another due to their frequencies and the proliferation of these radiations involve magnetic and electric forces from which arises the class name of electromagnetic radiation.

The characteristics linked to an electromagnetic wave's electrical component are important and the power needed to promote this transition from a lower energy state to a higher energy state equivalent to the electromagnetic radiation that initiates the transition.

So the higher the wavelength, the higher the energy and the lower the wavelength, the lower the energy.

Through the progression from cosmic radiation to ultraviolet and infrared regions and ultimately to radio frequencies, there is a migration towards lower energy regions.

A molecule may engross a distinct frequency, a significant energy transition occurs inside the molecule.

$$E = h\upsilon$$

Where:

E = energy, h = Planck's constant, υ = velocity

While a majority of regions of the electromagnetic range are utilised for the comprehension of matter in organic chemistry, organic chemists remain essentially preoccupied by energy absorptions in the regions from simply the infrared, ultraviolet, visible, radio frequency and microwave regions (Kumar, 2006)

2.6.1.7 Nature of electronic transitions

In the ultraviolet area, energy absorbed generates changes in the molecule's electronic energy. As a molecule absorbs energy, an electron is transferred into an unoccupied molecular orbital of higher potential energy from an occupied molecular orbital, where the electron is originally located.

Conventionally for a majority of molecules, the lowest-energy occupied molecular orbitals are the sigma orbitals (σ-orbitals) corresponding to sigma bonds (σ-bonds) of molecules, pi-orbitals (π-orbitals) corresponding to pi-bonds (π-bonds) are of greater energy followed by the non-bonding (n), pi- (π*) and sigma anti-bonding orbitals (σ*).

σ < π < n < π* < σ*, showing energies of different orbitals.

Saturated aliphatic alkanes undergo only σ → σ* transition, which is a high energy transition and involves very small wavelength ultraviolet light(below150 nm) but are able to undergo several other transitions depending on the functional groups present on compound, the organic molecules electron may undergo several possible transitions depicted below in increasing energies.

n → π* < n → σ* < π → π* < σ → π* < σ → σ*

Alkenes undergo σ → σ* and π → π* transitions, the π → π* are the lowest in energy between the two transitions and absorbs radiations with wavelengths ranging between 170 – 190 nm.

In saturated aliphatic ketones, the lowest energy transition involves the transfer of one electron of non-bonding electrons of oxygen to the relatively low-lying π* orbital (n → π* roughly at 280 nm) but is of low intensity as its symmetry forbidden and also π → π* transitions.

In conjugated dienes undergo the $\pi \rightarrow \pi^*$ transition, where two alkene groups orbitals combine to form new orbitals. In saturated compounds with a heteroatom bearing non-bonding pairs of electrons, $n \rightarrow \sigma^*$ transitions become available and are high in energy transitions.

In unsaturated molecules, $\pi \rightarrow \pi^*$ transitions can occur where alkenes and alkynes absorb light roughly around 170 nm but the occurrence of substituents on the molecule significantly influences their position. Carbonyl compounds and imines can undergo $n \rightarrow \pi^*$ transitions which absorb light around 280 – 300 nm as well as $\pi \rightarrow \pi^*$ transitions.

The transition of an electron from one energy level to another is accompanied by changes in the vibrational and rotational states of electron and leads to transitions between various vibrational and rotational levels of lower and higher energy electronic states. When a molecule absorbs ultraviolet or visible light of a defined energy, it's presumed that only one electron is excited and undergoes the transition while all the other electrons remain unaffected (Kumar, 2006).

2.6.1.8 Principles of absorption spectroscopy

The more molecules which are able to absorb light radiation of a defined wavelength, the more the magnitude of light that is absorbed resulting in a high peak intensity that is observed in the absorption spectrum.

Should there be only a small quantity of molecules which are capable to absorb the light radiation, there will be a small amount of light that is absorbed resulting in a low peak intensity on the absorption spectrum.

This constitutes the foundation of the Beer-Lambert law that says that the portion of incident radiation that is absorbed will be proportional to the number of absorbing molecule in its path used in absorption spectroscopy.

$$A = \varepsilon\, c\, l$$

Where:

A = Absorbance

ε = molar absorptivity or the molar extinction coefficient of the substance whose light absorption is under investigation.

c = concentration of the solute (Mol/L)

l = path length of the sample (cm).

Spectral measurements

UV/Vis spectra are customarily assessed in liquids that are very dilute in concentration and a vital condition in choosing a solvent to work with is that it must be translucent across the range of the wavelength that it is being observed in.

2.6.1.9 Solvent effects

Non-polar, very clear solvents for instance saturated hydrocarbons do not act together with the molecules of solute whether the molecules of the solute are in their excited or ground states and the absorption spectrum of an analyte is said to resemble an absorption spectrum of analyte in its gaseous state.

Though, polar solvents like alcohols can stabilise or destabilise a molecule's molecular orbitals whether the molecule is in its excited or ground state and the absorption spectrum of an analyte is said to vary a lot from an absorption spectrum of one in a hydrocarbon solvent.

Electronic spectroscopy can be used in estimating a maximum absorption of organic molecules such as conjugated trienes, dienes, polyenes and carbonyl compounds.

Also while detecting aromatic compounds with substituents with unshared electrons on the compounds. The effect of π-conjugation also affects electron-releasing and electron-withdrawing groups and interfere with detecting the presence of polycyclic aromatic compounds (Kumar, 2006).

2.7 Chromatography

It is an analytical technique that utilises numerous analytical procedures that are used for the separation of mixtures, in the case of the project the crude extracts, to isolate isolates present in a sample.

It embraces a number of processes that are based on differential partitioning of analyte components between two phases. One of these phases stays fixed in the systems and is known as the stationary phase and the other phase is called the mobile phase which moves over the surface of the fixed phase.

The movement of the phase causes a differential migration of the analyte components where more polar elements of an analyte are kept longer in the stationary phase than less polar elements of an analyte (Mlala, 2016).

2.7.1 Thin Layer Chromatography (TLC)

It is the simplest procedure that is utilised to get an overall picture of the number and types of metabolites that are present in a crude extracts. In essence it is used to visualise the chromatographic plant profile in crude extracts.

TLC is a hue of liquid chromatography where the stationary phase consists of a thin, uniform layer of dry, finely solid powder material like silica or alumina on an inert base such as aluminium or silica gel on a flat carrier such as a glass plate or a thick aluminium foil.

The mobile phase moves the solutes throughout the stationary phase and the polarity of the plant solutes of interest is vital to the selection of the mobile phase (Mlala, 2016).

The rate of travel by the solutes throughout the stationary phase is largely dependent on the mobile phase dissolving the solute and as the mobile phase moves the dissolved solute upward along the plate.

The rate of travel is also dependent on the sorbent resistance while the sorbent drags the solute away from solution it is dissolved in and back into the sorbent. Solutes travel the length of the plate in a stop-start manner while the solutes become repetitively absorbed and desorbed onto the sorbent (Ndlovu, 2007).

TLC is classed by its mechanism of separation: there is TLC that works by adsorption, which is the physical sorption of solutes onto the sorbent units and also by partition, which is the dissolving of solutes into a stationary liquid on the sorbent.

Also by means of ion exchange, it is the attraction of oppositely charged ions of solute and sorbent followed by the retention of solute onto the sorbent. Also by means of size exclusion or gel permission and that is the rejection or retention of solute based on shape or size of solute.

A spraying reagent is a visualisation reagent that is used to observe the number and types components and there are different types of reagents that can be prepared in order to visualise different types of components in plant sample extracts (Agostini-Costa et al, 2012).

An example of a spraying reagent is Anisaldehyde - sulfuric acid solution which is selective for anti-oxidants such as phenols, carbohydrates, prostaglandins, essential oil components, steroids, mycotoxins, glycosides, sapogenins, terpenes and antibiotics. The Anisaldehyde-sulfuric acid spraying reagent is said to be a universal reagent for natural products and aids in the identification of compounds (Agostini-Costa et al, 2012).

2.7.2 Column Chromatography

Column chromatography is an uncomplicated and practical procedure for purification and separation of compounds and is a useful technique for isolation of various secondary metabolites and bioactive compounds that are present in a sample.

Column chromatography is a solid-liquid chromatographic method where mobile phase is liquid and the stationary phase is solid. Common adsorbents utilised as the stationary phase in column chromatography are silica, magnesia, alumina, calcium phosphate and starch to list a few examples.

The solvent(s) that is required is chosen by the kind of solvent and adsorbent that is going to be used. Common solvents that used are methanol, hexane, benzene, toluene, hexane, ethyl acetate, etc.

The adsorbent is incorporated into the cylindrical tube by being made into a slurry called wet packing or poured into the cylindrical tube called dry packing with suitable solvent or solvent combination depending on the polarity needed and transferred into the cylindrical tube which is stopped at the end of the tube by either a porous disc or a piece of cotton wool.

The column is filled with a solvent that, due to the force of gravity, transports the sample throughout the column and the extract (analyte) that is to be used for isolation is then dissolved in an appropriate solvent and is poured into the column at the top of it and passes throughout the column.

As the analyte travels throughout the column, different components are adsorbed at separate areas along the stationary phase then the different constituents of the analyte are adsorbed onto stationary phase and gathered individually by the addition of more solvent at the top of the column and this process is called elution and the solvent the eluent.

As with different systems of chromatography, the variances in the movement rates of constituents throughout the stationary phase are resultant in the varied exit times at the bottom of the column for the different components of the sample extract.

The different fractions are then collected in separate beakers and distillation or evaporation of the solvent, result in the crystallisation of compound(s) which may pure or impure compound(s) which can be further analysed (Mlala, 2016; Ndlovu, 2007).

2.8 Biological Studies

2.8.1 Total Phenolic content

It is highly documented that many plants may have plenty phenolic compounds that have a hydroxyl group on an aromatic ring.

Phenolics disrupt chain oxidation reactions by donating chelating metals or a hydrogen-atom to the pro-oxidant, so therefore the phenolic compounds perform as reducing agents and anti-oxidants (El-Chaghaby, Ahmad & Ramis, 2014).

The basic mechanism of the assay is a redox reaction, where there is transfer of electrons in an alkaline medium from the phenolics to a solution that generates a blue chromophore made up of a phosphotungstic/ phosphomolybdenum complex with a maximum absorption that is concentration-dependent.

A UV/Vis spectrophotometer is utilised to detect the reduced Folin-Ciocalteau reagent in the range of 690 – 710 nm.

Polyphenols have come under deep attention in research due to their apparent health benefits and in literature are described to demonstrate anti-atherogenic, anti-carcinogenic , anti-thrombotic, anti-inflammatory, vasodilatory, immune-modulating-, analgesic- and anti-microbial effects. They are found in an array of fruits, seeds, flowers, vegetables, nuts, bark beverages and in some manufactured foods (Pongrakhananon. 2013).

2.8.2 Total Flavonoid content

The principle behind this assay involves an aluminium chloride ($AlCl_3$) colorimetric reaction in which $AlCl_3$ creates complexes that are acid stable with the carbon number 4 keto groups and with either carbon number 3 or carbon number 5 hydroxyl groups of flavonols and flavones.

Additionally, $AlCl_3$ creates complexes that are acid labile with orthodihydroxyl groups in A- or B-rings of flavonoids to gives the yellow colour visible upon completion of the reaction (Bhaigybati, Devi & Bag, 2014).

2.8.3 Ferric Reduction Antioxidant Power (FRAP)

The theory of the procedure has its basis on the Fe(III)-TPTZ (ferric tripyridyltriazine) complex being reduced to its Fe(II)-TPTZ (ferrous tripyridyltriazine) complex structure around and in the company of anti-oxidants.

The ferrous tripyridyltriazine (Fe(II)-TPTZ) complex contains a strong blue colour and its absorbance is read at 593 nm.

The assay assesses anti-oxidants on the basis of their activity as reductants in a redox-linked colourimetric reaction and it is regarded as a direct indicator of “total anti-oxidant power” of a substance (crude extracts or isolated compound) (Rebaya et al, 2014).

2.8.4 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

This test is based on the presumption that anti-oxidants react with DPPH that is reduced to yield DPPHH and as a result of the reaction an absorbance at 517 nm that can be assessed.

The procedure was created by Blois in 1958 with the view of determining the anti-oxidant activity of a substance by using a stable radical (DPPH).

DPPH is a stable radical having a delocalised extra electron around the molecule in its entirety, so that the molecules do not dimerise as the majority of F.R.s and this delocalisation is also the reason of the intense violet colour that has an absorption in EtOH around 520 nm.

Upon the reaction of DPPH with analyte in solution, the substrate can then contribute a hydrogen-atom to DPPH and the reduction results in the formation (DPPHH) with a reduction in the intensity of the violet colour.

DPPH is a fast, easy and universally utilised procedure to assess the capability of compounds to perform as hydrogen-donors or F.R. scavengers.

A main pro of the assay is that DPPH is permitted to react within the entire sample solution and enough time is allowed for the DPPH to react with the potential anti-oxidants even weak anti-oxidants as well.

DPPH is believed to be a true, precise, trouble-free and cheap test to assess the radical scavenging capability of anti-oxidants and anti-oxidant efficiency of compounds is evaluated at room temperature reducing risk that is associated with the thermal degradation of the molecules that are being evaluated.

Method has several restrictions in reflecting the partition of anti-oxidants in emulsion systems and is not handy for evaluating the anti-oxidant capability of plasma because of the proteins that have precipitated in the ethanolic medium (Kedare & Singh, 2011).

2.8.5 Lipid Peroxidation

The assay has its basis on N-methyl-2-phenylindole, which is a chromogenic reagent, reacting in the company of malondialdehyde (MDA) and/or 4-hydroxyalkenals at 45^{o}C.

Lipid peroxidation (L.P.) assay is an altered TBARS assay that is utilised to evaluate the lipid peroxides that are generated where egg yolk homogenates are used as the lipid-rich media.

L.P. is induced with the use of Iron sulphate (FeSO4). MDA, which is created by the oxidation of PUFA's that have reacted with two molecules of thiobarbituric acid (TBA) generating a chromogen that is pinkish-red in colour and it has a maximum absorbance at 532 nm which then can be assessed (Olorunnisola, Bradley & Afolayan, 2012).

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Chapter 3

Hippobromus pauciflorus

3.1 Genus Information

Figure 3.1: *Hippobromus pauciflorus* tree

Source: http://www.kumbulanursery.co.za accessed 15/04/2019

Sapindaceae Juss

The *Sapindaceae Juss* family is comprised of flowering plants that contain roughly 1900 species which are mainly found in tropical regions across the world. They usually develop in the under storey of forests either as shrubs or trees. These plants are characterised by their milky sap which may contain mildly toxic saponins (Adeyemi, Ogundipe & Olowokudejo, 2013).

Leaves are alternate or opposite, commonly estipulate, sessile or petiolate. Lamina is simple, palmatifid, pinnate or digitate. Inflorescences of the plant may be axillary or at terminal end of the plant which may be racemose in nature, cymose or flower solitary (Baker, 2009).

Flowers may be actinomorphic or zygomorphic in nature but can be bisexual or unisexual reproductively depending whether it need to be functionally male or functionally female to ensure the survival of the plant species (Baker, 2009).

Flowers generally have three to ten sepals which may be free and/or united in structure. On the other hand flowers have variable number of petals and stamens.

Sapindaceae usually have two locular anthers dehiscing longitudinally. They also have one to three but some as many as eight locular ovaries of superior position with one or two ovules per locule and usually one style.

These plants generally produce fruits that may be a drupe, capsule, berry or winged schizocarp consisting of two to three samaras and seeds that are often solitary but often are arillate and may contain an endosperm scantly or may be absent (Baker, 2009).

3.2 Literature Review

The leaves of *Hippobromus pauciflorus* are generally utilised by traditional healers for management of malaria and they are also crumpled and pressed into infected eyes while the root is acknowledged by Zulus as a love charm and it's also utilised in the management of dysentery and diarrhoea. Plant leaves extracts are used in the management of livestock diseases and pink eye (Pendota et al, 2009).

Pendota, Yakubu, Grierson and Afolayan (2009) reported that the acetone extracts of *H. pauciflorus* (bark, stem and roots) displayed significant activity against both Gram-positive and Gram-negative bacteria having MIC values that range between 0.1 – 10 mg/mL.

The methanol extracts exhibited strong activity against bacterial isolates with MIC values ranging between 0.5 – 10 mg/mL whilst the water extracts of the leaf exhibited activity against Gram-positive bacteria at 1.5 – 5.0 mg/mL.

Methanol extracts inhibited the growth of two fungi *Aspergillus niger and Penicillum notatum* by 78.70% – 100% at doses as low as 10 mg/ml. The acetone extracts displayed inhibitory activity against *Aspergillus niger* with an inhibitory percentage of 51.76% and 77.22% against *Penicillum notatum.* The bark water extract significantly inhibited the growth of *Penicillum notatum* with an inhibitory percentage of 81.02%.

Reported phytochemical screening of plant leaves has revealed the existence of tannins, cardiac glycosides, flavonoids, steroids, terpenes and saponins. The leaf extracts displayed various degrees of activity against inflammation, fever and pain.

The water extract of the plant at 50, 100 and 200 mg/kg body weight subdued the histamine- and carrageenan-induced inflammation in a way that was not dose-dependent in showing its anti-inflammation activity.

The water extract showed analgesic properties in the formal induced pain and tail flick pain models. The water extract was also shown to have dose-dependent anti-febrile properties at doses of 50 and 100 mg/kg body weight in the brewer's yeast injection test (Pendota et al, 2009).

Olorunnisola, Bradley and Grierson (2012) tested 70% acetone and ethanol extracts for anti-oxidant capability of the *H.pauciflorus* leaves by means of F.R. scavenging activities against 1, 1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing power activities (FRAP), 2, 2-azinobis-3-ethylbenzothizoline 6-sulfonic acid (ABTS) as well as nitric oxide and hydrogen peroxide scavenging properties and the ability to reduce or induce lipid peroxidation.

The acetone extract contained a greater amount of phenolic compounds than that of the ethanol extract while the quantities of flavonoids, proanthocyadins, tannins and flavonols in both extract tested were not different and the difference is postulated to be due to the relative polarities of the solvents used in their study.

The ferric reducing power of acetone and ethanol extracts grew with an increase of the concentration of extracts but the acetone extract exhibited greater reducing power than the ethanol extract and both extracts exhibited a higher reducing ability than Butylated hydroxytoluene (BHT) at the concentration of 0.1 mg/mL.

The authors also postulated that it may be most likely that the phenolic compounds in the plant could be acting as electron-donors thus decreasing the generation of F.R.s.

The ABTS percentage inhibition of acetone extract is higher than that of ethanol extract but significantly lower than that of Gallic acid at the maximum concentration of 75µg/mL with EC_{50} values ranging between 64.7 to 84.6 µg/mL.

The authors also postulated that the results of the study suggest that leaf could act in the protection of the physiochemical properties of the cellular membrane bilayer from serious free-radical cell dysfunction (Olorunnisola, Bradley & Afolayan, 2012).

The results of ABTS and DPPH radical scavenging activities assays revealed that the two extracts were quick and good F.R. scavengers and the ABTS activity of the acetone extract was greater than that of ethanol extract and BHT at all concentrations the plant was tested at.

The DPPH assays of both the ethanol and acetone extracts exhibited a pattern that was dose–responsive that was lower when compared to BHT and tannic acid. The percentage scavenging activity of the ethanol extract was greater than the acetone extract.

This difference in the results of the ABTS and DPPH scavenging activity may be due to different mechanisms of action of the two assays and that both extracts are able to scavenge hydrogen peroxide in a dose-dependent manner.

This property of the plant is accredited to the phenol compounds that are able to give electrons to hydrogen peroxide thus reducing it to water. EC_{50} values of both extracts and BHT were alike in hydrogen peroxide scavenging ability but were statistically less than the values got for tannic acid. Leaf extracts displayed significant inhibition of nitric oxide with EC_{50} values ranging from 62.4 to 78.0 µg/mL.

The observed activity is postulated to be due to phytochemical constituents in the plant. The study showed that the leaf extracts are a possible supply of existing and novel natural anti-oxidants which could aid in thwarting the progress of a range of oxidative stresses (Olorunnisola, Bradley & Afolayan, 2012).

Water extract of leaves was tested at doses of 50, 100 and 200 mg/kg body weight for 14 days. The extract was shown not to change the levels of red and white cells, large unstained cells, neutrophils, mean corpuscular volume, lymphocytes and platelets (Pendota, Yakubu, Grierson & Afolayan, 2009).

At doses of 100 and 200 mg/kg body weight, the extract significantly altered levels of haemoglobin and packed cell volume although the mean corpuscular haemoglobin decreased at 200 mg/kg body weight.

Levels of eosinophil's present in the serum were reported to have decreased at doses of 50 and 200 mg/kg body weight whereas basophil levels improved at all doses. Sadly, the extract is reported not to significantly change their liver- and kidney body weight proportions as well as levels of sodium chloride and total protein in the serum.

The activities of the enzymes alkaline phosphatase, gamma glutanyl transferase and alanine aminotransferase were not changed in the serum however the activity of aspartate amino transferase increased at the dose of 200 mg/kg body weight.

These authors concluded that the plant extract has minor and concentration-specific hepatotoxic, haemotoxic and nephrotoxic effects and that the plant might not be a safe oral medication at dosages tested (Pendota, Yakubu, Grierson & Afolayan, 2009).

3.3 Plant Description

It is a resinous tree that can grow up to 5 m high. It contains leaves that are simple, arranged in an alternate fashion and usually bears fruits, berries or seeds. It is largely dispersed in riverine thickets, mainly along stream banks and at the margins of evergreen forest of SA. (Pendota et al, 2009).

It is part of the Sapindaceae family and has common names of *uLathile* and *umFaz'onengxolo* in Xhosa, False Horsewood in English, *umFaz'othethayo* in Zulu and *BasterPerdepis* in Afrikaans. In SA it originates in the Eastern Cape and Western Cape but is also found in Limpopo, Mpumalanga and Kwa-Zulu Natal as well as Swaziland.

The plant leaves, stems and roots are utilised in treating influenza, headache, toothache, body cleansing, skin irritations, colds and bladder and kidney problems and as a blood purifier (Nzue, 2009).

3.4 Method and materials

3.4.1 Plant collection

The plant sample used for the experiments was obtained from Alice town in the Eastern Cape province of SA. It was found behind the University of Fort Hare Staff Housing Complex in Happy Rest suburb.

It is a tree that measures approximately two metres in height, with an off-white coloured stems and bark with small green to dark green leaves that bore no fruit, seeds or berries at the time of collection.

A voucher specimen (voucher nr: L.N/01) was identified by Professor Cupido from the Department of Botany of the University of Fort Hare at the Giffen Herbarium at the varsity.

3.4.2 Extraction

After the leaves were air-dried for two weeks, the leaves were separated from the stems. The leaves were weighed (550.620 g) on a weighing balance and blended with blender.

The blended sample was transferred to a large conical flask and the sample was subjected to sequential solvent extraction utilising different solvents i.e.: normal-Hexane (n-Hex), Dichloromethane (DCM), Ethyl acetate (EA), Methanol (MeOH) and 70% Ethanol (EtOH) in order of ascending polarity under continuous shaking with a mechanical shaker and the percentage yield would be calculated.

The solvent extracts were concentrated using a rotary evaporator and then dried using a fume cupboard.

Biological test: anti-oxidant assays

Several methods were used to investigate the anti-oxidant properties of the plant extract in order to determine its usefulness in disease prevention processes.

3.5 Phenol assay

3.5.1 Folin Ciocalteau Total Phenol protocol
Preparation of reagents

Folin Ciocalteau working reagent

1 mL of Foiln Ciocalteau was diluted with 9 mL distilled water (dH_2O).

Sodium Carbonate (Na_2CO_3) solution

7.5 g of sodium carbonate was dissolved with dH_2O and added up to 100 mL mark of a 100 mL volumetric flask.

Gallic acid stock solution

0.1 g of Gallic acid was dissolved with dH_2O and added up to 100 mL mark of a 100 mL volumetric flask.

The stock solution was further diluted to concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.563 µg/mL and a blank solution was also prepared for the standard curve.

Extract preparation

A 1 mg/mL sample of the extract (i.e.: n-Hex, DCM, EA, MeOH, 70 % EtOH) was prepared by dissolving 0.1 g of dry extract in 100mL of dH_2O).

Procedure

0.5 mL of the extract sample was mixed with 2 mL of Folin-Ciocalteau working reagent and left for 5 mins. in a test tube. Then 1 mL of Na_2CO_3 solution was added to the mixture and covered with aluminium foil for 60 mins.

The absorbance readings were recorded after incubation at 765 nm and expressed as microgram gallic acid equivalent per millilitre (µg GAE/mL). Procedure was conducted in duplicate, thrice for each extract sample. (Stankovic, 2011)

3.6 Total Flavonoid Assay

3.6.1 Flavones and Flavonols
Preparation of reagents

Aluminium chloride ($AlCl_3$) reagent

1.133 g of $AlCl_3$ and 0.40 g of sodium acetate were dissolved in a 100 mL of dH_2O,

Quercetin stock solution

0.01 g or 10 mg of quercetin was dissolved in 100 ml of dH_2O in a volumetric flask and further diluted to prepare 100 mL solutions with concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.563 μg/mL for the standard curve and an ethanolic blank

Extract preparation

A 1 mg/mL sample of the extract (i.e.: n-Hex, DCM, EA, MeOH, 70% EtOH) was prepared by dissolving 0.1 g of dry extract in 100 mL of dH_2O.

Procedure:

0.5 mL of the extract sample and 1.5 mL of quercetin standard solution were mixed together in a test tube, 1 mL of $AlCl_3$ reagent was added to the mixture and thus covered and incubated with Aluminium foil for 30 mins. The absorbance readings were recorded after incubation at 430 nm and expressed as as quercetin equivalents per milligram of extract (QE/mg extract). Procedure was conducted in duplicate, thrice for each extract sample. (Stankovic, 2011)

3.7 Ferric Reducing Anti-oxidant Power (FRAP) assay
Preparation of reagents

Hydrochloric acid (HCl) 40mM solution

0.146 mL of concentrated HCl was mixed with 100 ml of dH_2O.

Acetate Buffer Solution (300mM)

3.1 g of sodium acetate trihydrate was dissolved in 500 mL of dH_2O, to that solution 16 mL of glacial acetate acid was added and dH_2O was added up to 1000 mL.

Iron chloride (FeCl3) solution (20mM)

1.1 g of $FeCl_3$ was dissolved in 200 mL of dH_2O and stored at room temperature until use.

Tripyridyltriazine (TPTZ) solution

0.156 g of TPTZ was dissolved in 50 mL of 40 mM HCl. Solution is made to be used on the day the assay is conducted.

FRAP reagent

100 mL of the acetate buffer solution as well as 10 mL of fresh TPTZ solution and 10 mL were combined to form the FRAP reagent.

Ascorbic acid stock solution

0.1 g of Ascorbic acid was dissolved in 100 mL of dH_2O and added up to 100 ml mark of a volumetric flask. It was serial-diluted to prepare further 100mL solutions with concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.563 μg/mL for the standard curve and an ethanolic blank.

Extract preparation

A 1 mg/mL sample of the extract (i.e.: n-Hex, DCM, EA, MeOH, 70 % EtOH) was prepared by dissolving 0.1 g of dry extract in 100 mL of dH_2O.

Procedure:

0.1 mL of the extract sample was mixed with freshly prepared FRAP reagent in a test tube, then covered with Aluminium foil and incubated for 15 mins at room temperature. The absorbance readings were recorded after incubation at 517 nm; the linear regression equation of the sample curve was used to extrapolate anti-oxidant activity and expressed as Ascorbic acid equivalent per mL (AAE/mL) (Guo, Yang, Wei, Li & Jiang, 2003).

3.8 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH solution

0.01 g of DPPH was dissolved in 500 mL of MeOH.

Ascorbic acid stock solution

0.1 g of Ascorbic acid was dissolved in 100 mL of dH_2O and added up to 100 ml mark of a volumetric flask. It was double-diluted to prepare further 100mL solutions with concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563 µg/mL for the standard curve and a methanolic blank.

Extract preparation

A 1 mg/mL sample of the extract (i.e.: n-Hex, DCM, EA, MeOH, 70 % EtOH) was prepared by dissolving 0.1 g of dry extract in 100 mL of dH_2O. By means of serial-dilutions, further 100 mL solutions of the extract sample with differing concentrations of: 0.5, 0.25, 0.125, 0.06, 0.03 mg/mL were prepared.

Procedure:

1 mL of the extract sample was transferred into a test tube, and then 3 mL of DPPH solution was added and covered with Aluminium foil and incubated at room temperature for 30 mins. The absorbance readings were recorded at 517 nm.

Percentage inhibition of DPPH radical was calculated using the equation below, where the curves were plotted and the IC_{50} values were determined. (Stankovic, 2011)

$$\% \text{ Inhibition} = \frac{A(blank) - A(sample)}{A(blank)}\ x\ 100$$

3.9 Lipid peroxidation (300 µg/mL and 150 µg/mL)
Preparation of reagents

Thiobarbituric-Trichloroacetic acid (TBA-TCA) reagent

0.392 g of Thiobarbituric acid (TBA) was dissolved in 75 mL of 0.25M HCl. To the solution, 5 g of Trichloroacetic acid (TCA) was added then dH_2O was added to 100 mL mark of the volumetric flask.

0.07 M Iron sulphate ($FeSO_4$) solution

0.5316 g of $FeSO_4$ was dissolved in 50 mL of dH_2O.

Extract preparation

A 1 mg/mL sample stock solution of the extract (i.e.: n-Hex, DCM, EA, MeOH, 70% EtOH) was prepared by dissolving 0.1 g of dry extract in 100 mL of dH_2O.

It was further diluted to prepare 100 mL extract samples that had concentrations of 300 and 150 µg/mL by pipetting 3 mL and 1.5 mL stock extract solution and completed dH_2O up to 10 mL mark.

Procedure:

0.5 mL of the egg yolk homogenate (10% v/v) and 0.1 mL of the extract sample were mixed together in a test tube, to the mixture 0.05 mL of $FeSO_4$ solution was added to induce oxidative stress.

The mixture was incubated at 37^oC for 30 mins in an oven. 3 mL of TBA-TCA reagent was added and centrifuged at 3000 rpm for 15 mins. A pinkish-red chromogen formed and absorbance readings were recorded at 532 nm with the percentage inhibition then calculated using the equation below. (Ruberto, Baratta , Deans & Dorman, 2000)

$$\% \text{ Inhibition} = \frac{A(blank) - A(sample)}{A(blank)}\ x\ 100$$

Where:

$A_{(blank)}$ = absorbance of oxidised egg yolk without extract.

$A_{(sample)}$ = Absorbance of oxidised egg yolk with extract.

3.10 Statistical Analysis

GraphPad Instat was used for all data analysis. One-way Anova followed by Tukey-Kramer multiple comparison test, was used to determine difference in effects of extracts. All the results are presented as Mean ± SEM and considered to be statistically significant at $p < 0.05$.

3.11 Results and discussions

Solvent extraction

After the sequential solvent extraction of the leaves was concluded, different masses of crude extracts were obtained with DCM (0.817 g) yielding the smallest mass and the 70% EtOH (120.196 g) extract yielding the largest mass.

The masses followed this order in terms of masses: DCM < MeOH < EA < Hex < EtOH (Table 3.1).

Table 3.1: Showing results of solvent extraction of the leaves.

Extract	**Physical appearance**	**Mass of extract (grams)**	**Percentage yield % (w/w)**
n-Hexane	Dark green and oily	21.996	18.30
Dichloromethane	Green and oily	0.817	0.49
Ethyl Acetate	Dark brown and solid	2.829	1.70
Methanol	Green and oily	1.270	1.06
70% Ethanol	Dark brown and solid	120.196	21.83

Total phenolic content

The total phenolic content of the extracts was performed using the Folin Coicalteau reagent. Results expressed as microgram gallic acid equivalent per millilitre.

The MeOH extract (133.019 ± 0.003 µgGAE/mL) exhibited the largest total phenolic content while the EtOH extract (13.981 ± 0.001 µgGAE/mL) exhibited the least total phenolic content.

The relative abundance of phenolic acid in the extracts was significantly ($p<0.001$) different in the different extracts as follows: EtOH < DCM < EA < Hex < MeOH and all extracts besides the hexane extract were statistically significant i.e. $p < 0.05$ (Figure 3.2).

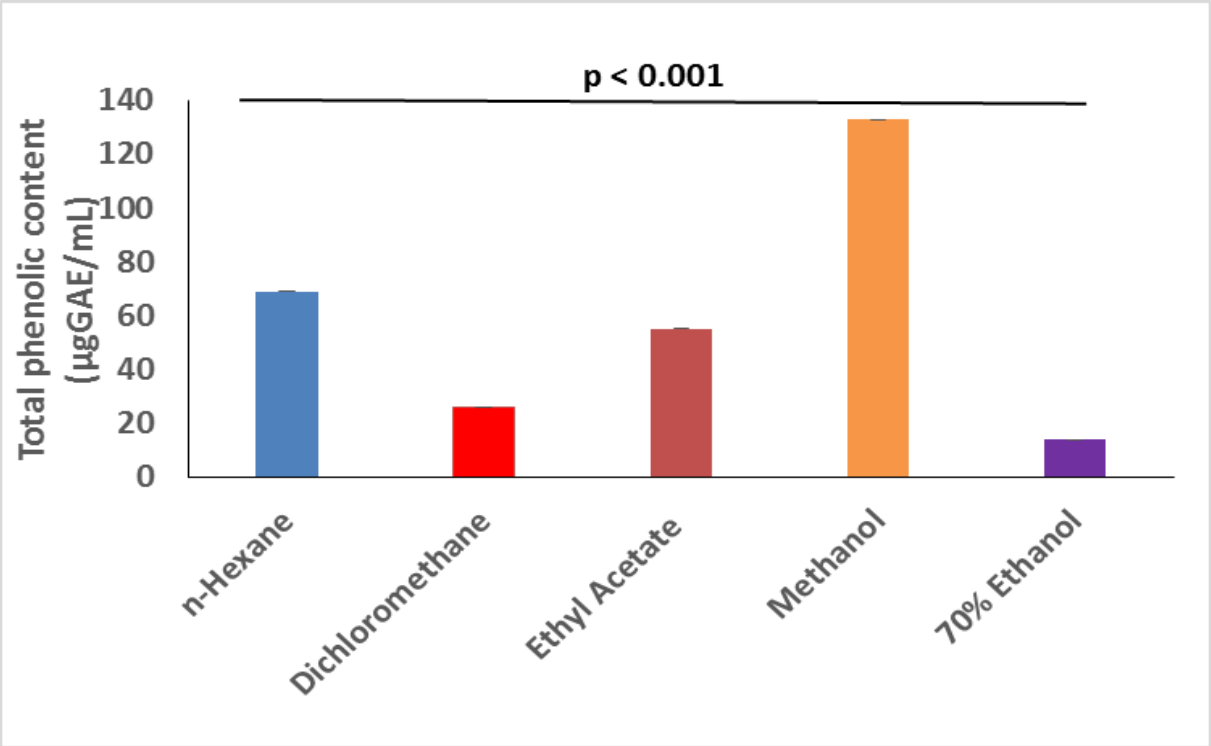


Figure 3.2: Total phenolic content of extracts of *H. pauciflorus*

Total Flavonoid Content

The principle behind this assay involves an aluminium chloride colorimetric reaction in which aluminium chloride forms acid stable complexes. Results were presented as microgram quercetin equivalents per milligram of extract (µgQE/mg extract).

The MeOH extract (46.005 ± 0.001 µgQE/mg extract) exhibited the largest flavonoid content while the acetyl acetate extract (19.000 ± 0.008 µgQE/mg extract) exhibited the least content.

The order of relative abundance is as follows: EA < Hex < EtOH < DCM < MeOH and all extracts were statistically significant i.e. $p < 0.05$. Results showed that the difference in flavonoid content was statistically ($p < 0.001$) different between extracts (Figure 3.3).

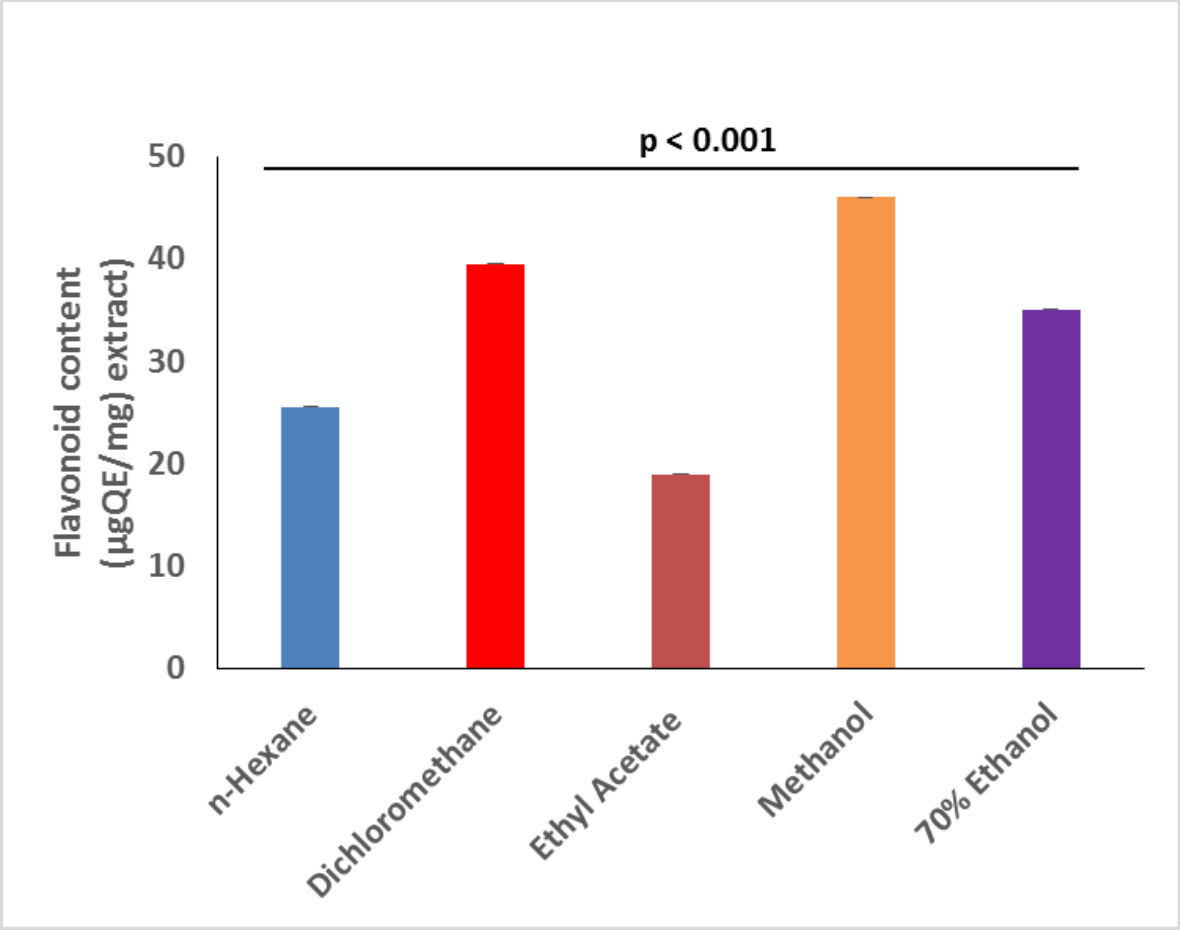


Figure 3.3: Flavonoid content of extracts of *H. pauciflorus*

FRAP assay

The principle of this method is based on the reduction of a ferric tripyridyltriazine complex (Fe (III)-TPTZ) to its ferrous tripyridyltriazine complex form in the presence of anti-oxidants and the change in the colour intensity indicated antioxidant capacity. The results were measured in ascorbic acid equivalent per mg of extract (AAE/mg of extract).

The EA extract (10.284 ± 0.014 AAE/mL extract) displayed the least FRAP ability while the Hex extract (204.705 ± 0.119 AAE/mg extract) the highest.

The order of activity was as follows: EA < DCM < MeOH < EtOH < Hex. The total antioxidant capacity of the extracts were significantly ($p < 0.001$) different between extracts (Figure 3.4).

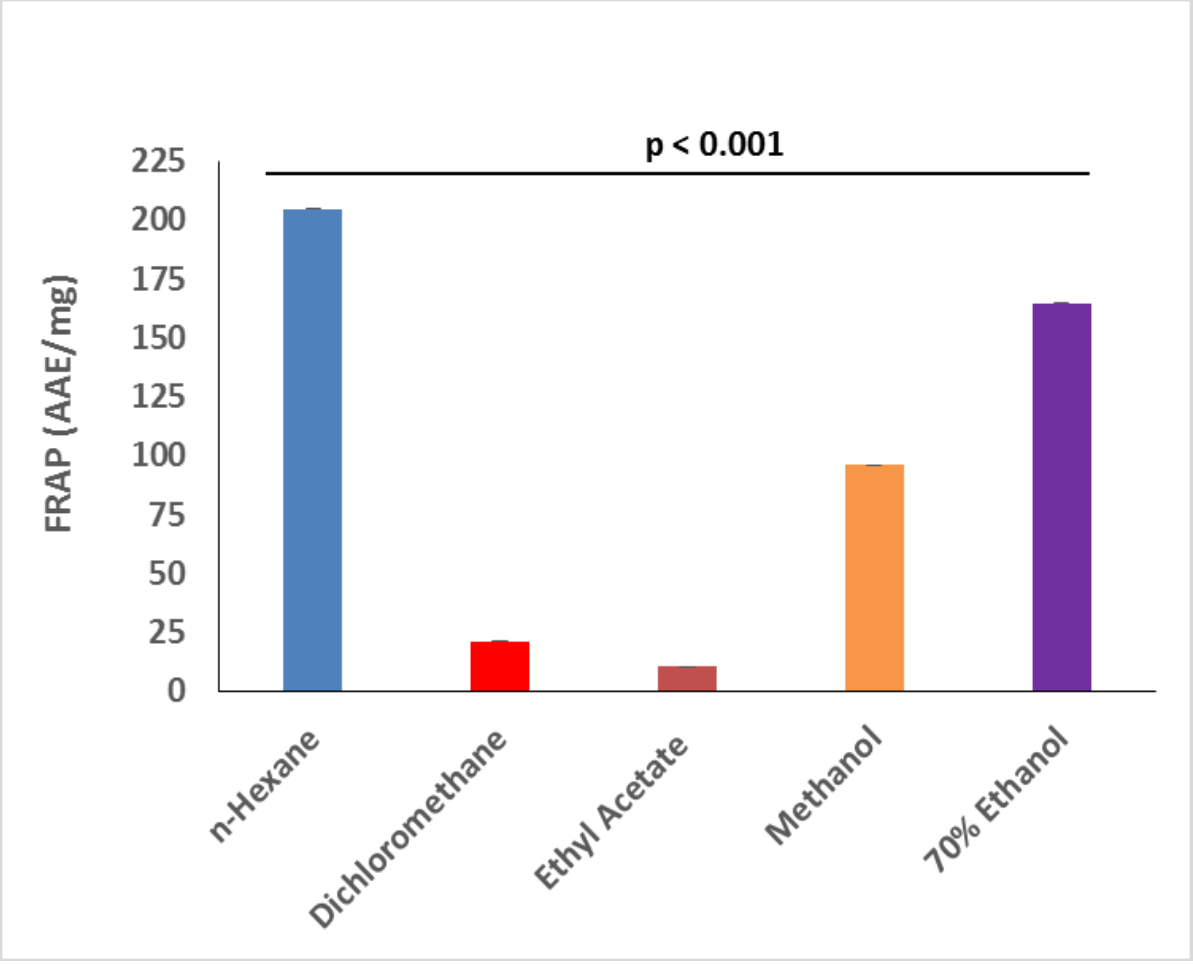


Figure 3.4: Total antioxidant capacity as measured by ferric reducing antioxidant power (FRAP) assay of *H. pauciflorus*.

DPPH assay

The assay is based on the assumption that anti-oxidants react with 2, 2-diphenyl-1-picrylhydrazyl (DPPH). It is a stable F.R., thus is reduced to the DPPHH and as a consequence of the reaction an absorbance at 517 nm can be measured.

Table 3.2: Showing the % Inhibition and IC_{50} values of the extracts.

Concentration (mg/mL)	**n-Hex % Inhibition**	**DCM % Inhibition**	**EA % Inhibition**	**MeOH % Inhibition**	**EtOH % Inhibition**
0.03	42.804	0.554	0.369	22.140	30.812
0.06	44.557	1.199	1.199	21.125	30.074
0.125	64.391	1.568	1.476	36.808	49.077
0.25	82.749	5.351	3.413	54.244	71.863
0.5	86.162	16.328	4.889	75.369	84.225
1	85.424	13.1	0.0923	83.303	86.624
IC_{50} value (mg/mL)	$3.594x10^{-3}$ ± 0.133	2600.269 ± 0.039	1.068x1046 ± 0.032	0.185 ± 0.019	0.011 ± 0.002

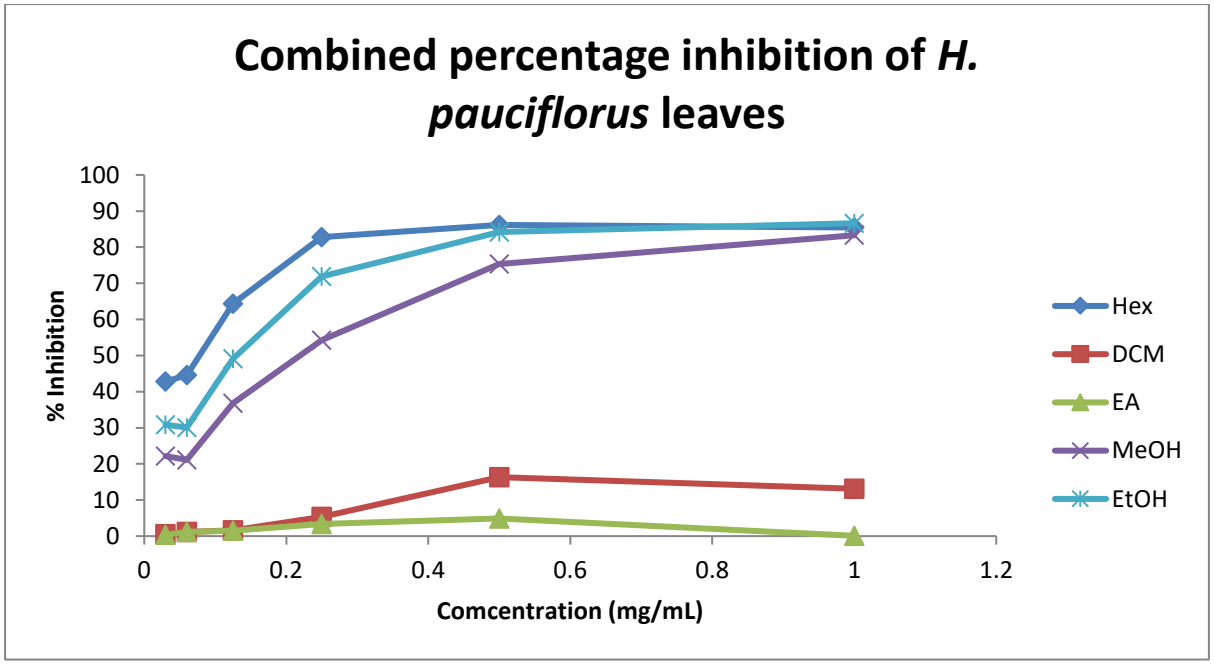
The percentage inhibition of the Hex extract ranged between 42.804% and 86.162% showing an ability to scavenge the DPPH radical, with an IC_{50} value of $3.594x10^{-3}$ mg/mL and also was statistically not significant (Table 3.2).

The percentage inhibition of the DCM ranged between 0.554% and 13.1%, with an IC_{50} value of 2600.269 ± 0.039 mg/mL showing an inability to scavenge the DPPH radical and also was statistically not significant (Table 3.2).

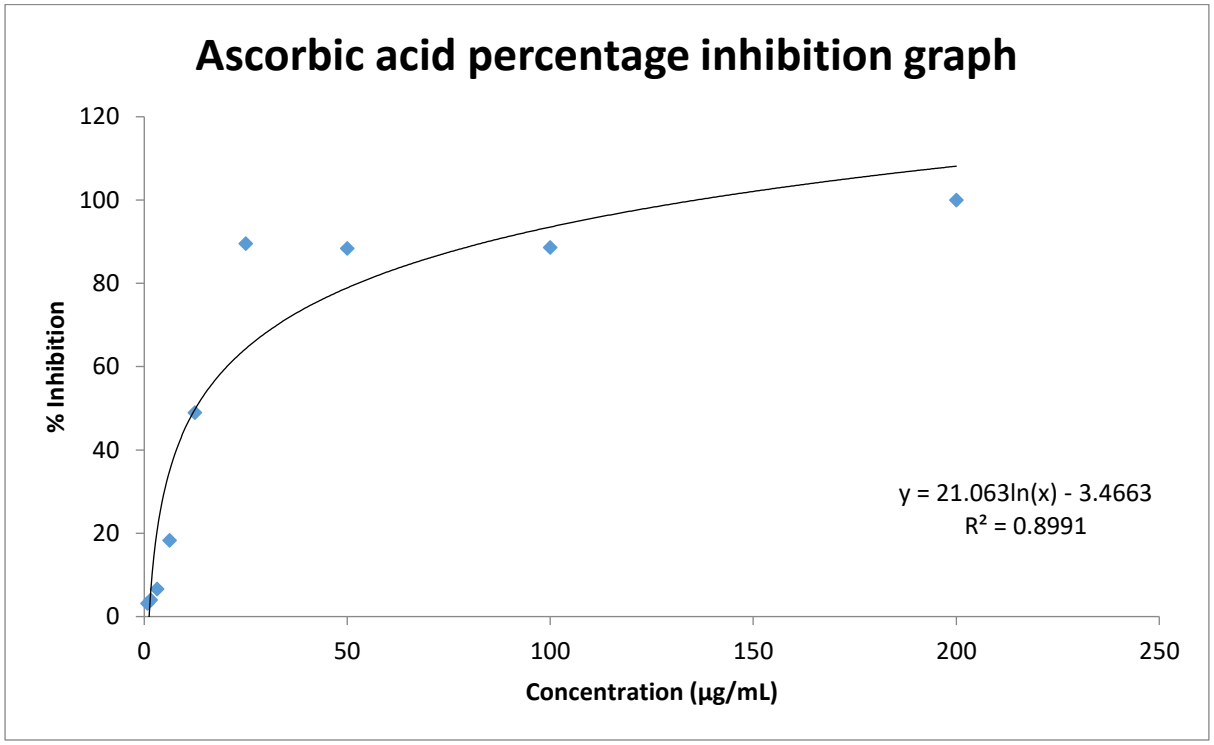
The percentage inhibition of the EA extract ranged between 0.092% and 4.889%, with an IC_{50} value of $1.068x10^{46}$ ± 0.032 mg/mL showing an inability to scavenge the DPPH radical and also was statistically not significant (Table 3.2).

The percentage inhibition of the MeOH extract ranged between 21.125% and 83.303% showing an ability to scavenge the DPPH radical, with an IC_{50} value of 0.185 ± 0.019 mg/mL and also was statistically not significant (Table 3.2).

The percentage inhibition of the EtOH ranged between 30.074% and 86.624% showing an ability to scavenge the DPPH radical, with an IC_{50} value of 0.011 ± 0.002 mg/mL and also was statistically not significant (Table 3.2).



Graph 1: Showing % Inhibition of *H.pauciflorus* extracts in DPPH assay



Graph 2: Showing standard curve % Inhibition of stock Ascorbic acid extracts in DPPH assay

Lipid peroxidation (150 µg/mL)

All the extract were statistically significant with two inhibiting the peroxidation of lipids with the DCM extract -75.000 ± 0.119%) showing the highest inhibition percentage while the Hex extract (-41.667 ± 0.021005%) showing the lowest inhibition percentage while the MeOH, Hex and EtOH extracts showed no inhibition.

The order of percentage inhibition is as follows: EtOH < MeOH < Hex < EA < DCM (Table 3.3)

Table 3.3: Showing results of lipid peroxidation (150 µg/mL) inhibition.

Extract	**% Inhibition**	**P-value**	**Significance**
n-Hexane	22.789 ± 0.030	$4.998x10^{-4}$	Significant***
Dichloromethane	-75.000 ± 0.119	$4.694x10^{-2}$	Significant*
Ethyl Acetate	-41.667 ± 0.021	$1.866\ x10^{-2}$	Significant*
Methanol	7.483 ± 0.004	$3.434\ x10^{-2}$	Significant*
70% Ethanol	21.769 ± 0.028	$2.180\ x10^{-2}$	Significant*

*: p<0.05, **: p<0.01, ***: p<0.001

Lipid Peroxidation (300 µg/mL)

Only the DCM extract (-3.051 ± 0.018%) displayed inhibition but the rest didn't and only the EA extract was statistically significant and the rest were not statistically significant. The order of percentage inhibition is as follows: MeOH < EtOH < Hex < EA < DCM (Table 3.4).

Table 3.4: Showing results of lipid peroxidation (300 µg/mL) inhibition.

Extract	**% Inhibition**	**P-value**	**Significance**
n-Hexane	5.593 ± 0.013	$9.553x10^{-1}$	Not significant
Dichloromethane	-3.051 ± 0.018	$5.522x10^{-1}$	Not significant
Ethyl Acetate	0.847 ± 0.007	$1.526x10^{-2}$	Significant*
Methanol	16.102 ± 0.014	$1.857x10^{-1}$	Not significant
70% Ethanol	8.983 ± 0.023	$6.254x10^{-1}$	Not significant

*: p<0.05, **: p<0.01, ***: p<0.001

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Chapter 4

Pentanisia prunelloides

4.1 Genus Information

Figure 4.1: *Pentanisia prunelloides*

Source: https://wildflowernursery.co.za/wp-content/uploads/2015/11/Pentanisia_prunelloides_Dullstroom_500X5001.jpg accessed 15/04/2019.

Pentanisia prunelloides belongs to the Rubiaceae family that consists of 630 genera and about 13000 species which constitutes it as the fourth largest family of flowering plants coming after the Asteraceae, Orchidaceae and Fabaceae families.

This family is mainly made up of trees and shrubs but also includes perennial and annual herbs or lianas and constitutes a vital part of all tropical woody foliage, particularly rain forest understory. They are found in warm tropical regions (Karou, Tchacondo, Ilboudo & Simpore, 2011).

The leaves of the family are normally opposite or whorled with interpetiolar stipules that are sometime leaf-like. The family generally produces flowers that have four to five petals that are united to form a tube and generally produce fruits which are capsular, drupes or berries.

Some species of the Rubiaceae family have had their isolated compounds researched and discovered to have a diversity of biological activities which include anti-inflammatory, anti-tumour, anti-microbial, larvicidal, anti-oxidant, anti-ulcer and hepatoprotective.

Many species have exhibited antimalarial, antimicrobial, anti-hypertension, anti-diabetic, anti-oxidant and anti-inflammatory activities which have led to widespread usage of the plants from this family (Karou, Tchacondo, Ilboudo & Simpore, 2011).

The presence of phytochemicals such as sterols, alkaloids, proanthocyanidins, terpenes, tannins, flavonoids, terpenoids, alkaloids, saponins, coumarins, carotenoids and anthracenosides has been documented (Kala, 2015).

4.2 Literature Review

There are fifteen species of Pentanisia that are found in tropical Africa with the last three found in Southern Africa.

P.arenaria, P.aurongyne, P.calcicola, P. confertifola, P.foetida, P. logipenduculata, P.longituba, P. monticola, P.ouranogyne, P.paruiflora, P.owranogyne, P.procumbens, P.prunelloides, P.shweinfurthii and P. angustifolia last three occurring in SA (Ndlovu, 2007).

The root of *P.prunelloides* is the part of the plant that is used most frequently for traditional medicinal purposes though the leaves are also used occasionally.

It is reported to cure a variety of ailments such as fever, rheumatism, sore joints, wounds, chest pains, colds, stomach pain, burns, heartburn, swellings, vomiting, pain, toothache, tuberculosis, blood impurities, haemorrhoids and snakebites, venereal diseases, itch, palpitation, bladder and kidney complaints, tired legs, rheumatoid arthritis, sprains, inflammation, gangrenous rectitis and ulcers (Ndlovu, 2007).

The Zulus make an infusion of the root to be used as an enema for stomach pain. They also apply either a poultice of the leaf or a hot decoction of the plant to painful swellings, rheumatic parts, sprains and sores.

The root is used internally and externally to halt the emetic effects of "*infuzana*" and also as a pile remedy. The leaf poultice can also be applied to the lower abdomen to remove a retained placenta in both humans and animals.

The Southern Sotho soak a limb that has boils in a decoction of the root and is believed to help with the discharge of pus, while they also apply the plant lotion to the breast of a recently confined mother and to bathe patients (Ndlovu, 2007).

The Xhosa people use a decoction of the root to aid in the alleviation of distension of the abdomen, as well as a Tuberculosis remedy and in the treatment of gonorrhoea and infertility. This plant seems to be used as an anti-inflammatory medicine. Secondary metabolites such as anthraquinones, Indole alkaloids and terpenoids have been elucidated from this plant species and the presence of alkaloids in some members of the family is well acknowledged (Ndlovu, 2007).

Miya et al. (2016) previously worked with the leaves and rhizome of *P.prunelloides* and the dried plant material was extracted using MeOH, EtOH and water as solvents.

The water extract of the rhizome was found to contain saponins, tannins, flavonoids, glycosides, steroids and terpenoids and ditto for the MeOH extract and EtOH extract additionally alkaloids where present in the EtOH extract.

In the leaf, they reported the existence of saponins, glycosides, flavonoids, tannins, alkaloids, terpenoids and steroids in the EtOH and MeOH extracts whilst the water contained all besides alkaloids. The water extract of the leaf and rhizome was tested for acute toxicity and found to cause no mortality at doses up to 5000 mg/kg and the LD_{50} was approximated up to 5000 mg/kg p.o.

The water extract of both leaf and rhizome were investigated to determine if they could reduce the size of a carrageenan-induced paw oedema in rats at dosages of 250 and 500 mg/kg. Both extracts decreased the oedema size after 1, 2, 3 and 4 hours and the rhizome water extract was more effective at reducing the oedema size than the leaf extract.

The water extract of both leaf and rhizome were investigated to determine if they could decrease paw-licking in mice, which was formalin-induced at doses of 500 and 1000 mg/kg. Both extracts reduced paw-licking in the mice when compared to the vehicle in the first and second stages (Miya et al, 2016)

Mpofu, Msagati and Krause (2014) had worked with *P.prunelloides* rhizomes, the ground plant material was extracted using water and MeOH and liquid-liquid extraction was performed to obtain EA and Chloroform fractions. Diosgenin and Oleanolic acid have been isolated from *P.prunelloides.*

The cytotoxicity of *P.prunelloides* was assessed using the Brine Shrimp Lethality test and found the water and MeOH extracts showed a high level of biological activity with a LC_{50} value of 5.6.

They also reported that *P.prunelloides* was highly effective as anti-oxidant against the DPPH radical. *P.prunelloides* is traditionally also used in synergy with *Elephantorrhiza elephantina* by healers. Anthocyanidins have been found to be present in the rhizome after phytochemical screening (Mpofu, Msagati & Krause, 2014)

Muleya, Sipamla, Mtunzi and Mutatu (2015) had worked with *P.prunelloides* where an acetone extract was prepared, reconstituted in 70% acetone and underwent sequential extraction to yield n-hex, DCM, EA and MeOH fractions.

Their study demonstrates that the crude extract as well as the fractions of *P.prunelloides* demonstrated weak anti-bacterial activity with a MIC at 625 µg/ml against *E. faecalis*.

The study demonstrated that *P.prunelloides* has high anti-oxidant activity with similar levels of activity against DPPH and lipoxygenase radicals. *P.prunelloides* was reported to possess moderate to good activity anti-microbial activity (Muleya, Sipamla, Mtunzi & Mutatu, 2015).

4.3 Plant Description

Pentanisia prunelloides goes by the common names of Wild Verbana or broad leaved Pentanisia in English, Sooibrandbossie in Afrikaans, iciMamlilo in Zulu, Is'Cikamlilo in Xhosa and liciMamlilo in siSwati.

It is an erect, perennial herb that grows to be roughly 30 cm tall, it has stout hairy stems sprouting from a tuberous root and hairy leaves that are oblong in shape and in pairs. Plant yields pale purple flowers in early spring (around September) that are densely grouped on the branch ends.

The plant is dormant during winter while the aerial parts dry and the tuber remains underground until the first few rains of spring. In SA, it is dispersed across the provinces of the Gauteng, Kwa-Zulu Natal, Mpumalanga, Eastern Cape, Limpopo and North West and also in Lesotho. (Ndlovu, 2007)

4.4 Method and materials

4.4.1 Plant collection

Roots of the plant were obtained from Mr Mahlakata, a traditional healer in Mzizangwa village in the province of the Eastern Cape in SA.

4.4.2 Extraction preparation

After the roots were air-dried for 14 – 21 days, the roots were weighed (1590.361 g) on a weighing balance and ground with a mill.

The ground sample was transferred to a large conical flask and from there the sample underwent sequential solvent extraction using different solvents i.e.: normal-Hexane (n-Hex), Dichloromethane (DCM), Ethyl acetate (EA), Methanol (MeOH) and 70% Ethanol (EtOH) in order of ascending polarity under continuous shaking with a mechanical shaker and the percentage yield was calculated.

The solvent extracts were concentrated using a rotary evaporator and then dried in a fume cupboard.

Biological test: anti-oxidant assays

Several methods are utilised to investigate the anti-oxidant properties of the plant extract in order to determine its usefulness in disease prevention processes.

4.5 Phenol assay

4.5.1 Folin Ciocalteau Total Phenol protocol
Preparation of reagents

Folin Ciocalteau working reagent

1 mL of Foiln Ciocalteau was diluted with 9 mL distilled water (dH_2O).

Sodium Carbonate (NaCO3) solution

7.5 g of sodium carbonate was dissolved with dH_2O and added up to 100 mL mark of a 100 mL volumetric flask.

Gallic acid stock solution

0.1 g of Gallic acid was dissolved with dH_2O and added up to 100 mL mark of a 100 mL volumetric flask.

The stock solution was further diluted to concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.563 µg/mL and a blank solution was also prepared for the standard curve.

Extract preparation

A 1 mg/mL sample of the extract (i.e.: n-Hex, DCM, EA, MeOH, 70 % EtOH) was prepared by dissolving 0.1 g of dry extract in 100 mL of dH_2O).

Procedure

0.5 mL of the extract sample was mixed with 2 mL of Folin-Ciocalteau working reagent and left for 5 mins. in a test tube. Then 1 mL of $NaCO_3$ solution was added to the mixture and covered with aluminium foil for 60 mins.

The absorbance readings were recorded after incubation at 765 nm and expressed as microgram gallic acid equivalent per millilitre (µgGAE/mL). Procedure was conducted in duplicate, thrice for each extract sample. (Stankovic, 2011)

4.6 Total Flavonoid Assay

4.6.1 Flavones and Flavonols
Preparation of reagents

Aluminium chloride (AlCl3) reagent

1.133 g of $AlCl_3$ and 0.40 g of sodium acetate were dissolved in a 100 mL of dH_2O,

Quercetin stock solution

0.01 g or 10 mg of quercetin was dissolved in 100 ml of dH_2O in a volumetric flask and further diluted to prepare 100 mL solutions with concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.563 µg/mL for the standard curve and an ethanolic blank

Extract preparation

A 1 mg/mL sample of the extract (i.e.: n-Hex, DCM, EA, MeOH, 70% EtOH) was prepared by dissolving 0.1 g of dry extract in 100 mL of dH_2O.

Procedure:

0.5 mL of the extract sample and 1.5 mL of quercetin standard solution were mixed together in a test tube, 1 mL of $AlCl_3$ reagent was added to the mixture and thus covered and incubated with Aluminium foil for 30 mins.

The absorbance readings were recorded after incubation at 430 nm and expressed as microgram quercetin equivalents per milligram of extract (µgQE/mg extract). Procedure was conducted in duplicate, thrice for each extract sample. (Stankovic, 2011)

4.7 Ferric Reducing Anti-oxidant Power (FRAP) assay
Preparation of reagents

Hydrochloric acid (HCl) 40 mM solution

0.146 mL of concentrated HCl was mixed with 100 ml of dH_2O.

Acetate Buffer Solution (300 mM)

3.1 g of sodium acetate trihydrate was dissolved in 500 mL of dH_2O, to that solution 16 mL of glacial acetate acid was added and dH_2O was added up to 1000 mL.

Iron chloride ($FeCl_3$) solution (20 mM)

1.1 g of $FeCl_3$ was dissolved in 200 mL: of dH_2O and stored at room temperature until use.

Tripyridyltriazine (TPTZ) solution

0.156 g of TPTZ was dissolved in 50 mL of 40 mM HCl. Solution is made to be used on the day the assay is conducted.

FRAP reagent

100 mL of the acetate buffer solution as well as 10 mL of fresh TPTZ solution and 10 mL were combined to form the FRAP reagent.

Ascorbic acid stock solution

0.1 g of Ascorbic acid was dissolved in 100 mL of dH_2O and added up to 100 ml mark of a volumetric flask. It was double-diluted to prepare further 100mL solutions with concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.563 μg/mL for the standard curve and an ethanolic blank.

Extract preparation

A 1 mg/mL sample of the extract (i.e.: n-Hex, DCM, EA, MeOH, 70% EtOH) was prepared by dissolving 0.1 g of dry extract in 100 mL of dH_2O.

Procedure:

0.1 mL of the extract sample was mixed with freshly prepared FRAP reagent in a test tube, then covered with Aluminium foil and incubated for 15 mins at room temperature. The absorbance readings were recorded after incubation at 517 nm; the linear regression equation of the sample curve was used to extrapolate anti-oxidant activity and expressed as Ascorbic acid equivalent per mL (AAE/mL) (Guo, Yang, Wei, Li & Jiang, 2003).

4.8 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH solution

0.01 g of DPPH was dissolved in 500 mL of MeOH.

Ascorbic acid stock solution

0.1 g of Ascorbic acid was dissolved in 100 mL of dH_2O and added up to 100 ml mark of a volumetric flask. It was double-diluted to prepare further 100 mL solutions with concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563 µg/mL for the standard curve and a methanolic blank.

Extract preparation

A 1 mg/mL sample of the extract (i.e.: n-Hex, DCM, EA, MeOH, 70% EtOH) was prepared by dissolving 0.1 g of dry extract in 100 mL of dH_2O. By means of double-dilutions, further 100 mL solutions of the extract sample with differing concentrations of: 0.5, 0.25, 0.125, 0.06, 0.03 mg/mL were prepared.

Procedure:

1 mL of the extract sample was transferred into a test tube, and then 3 mL of DPPH solution was added and covered with Aluminium foil and incubated at room temperature for 30 mins. The absorbance readings were recorded at 517 nm.

Percentage inhibition of DPPH radical was calculated using the equation below, where the curves were plotted and the IC_{50} values were determined (Stankovic, 2011).

$$\% \text{ Inhibition} = \frac{A(blank) - A(sample)}{A(blank)}\ x\ 100$$

4.9 Lipid peroxidation (300 µg/mL and 150µg/mL)
Preparation of reagents

Thiobarbituric-Trichloroacetic acid (TBA-TCA) reagent

0.392 g of Thiobarbituric acid (TBA) was dissolved in 75 mL of 0.25M HCl. To the solution, 5 g of Trichloroacetic acid (TCA) was added then dH_2O was added to 100 mL mark of the volumetric flask.

0.07 M Iron sulphate ($FeSO_4$) solution

0.5316 g of $FeSO_4$ was dissolved in 50 mL of dH_2O.

Extract preparation

A 1 mg/mL sample of the extract (i.e.: n-Hex, DCM, EA, MeOH, 70% EtOH) was prepared by dissolving 0.1 g of dry extract in 100 mL of dH_2O and further diluted to prepare 100 mL extract samples that had concentrations of 300 and 150 µg/mL by pipetting 3 mL and 1.5mL stock extract solution respectively and filled with dH_2O up to 10 mL mark.

Procedure:

0.5 mL of the egg yolk homogenate (10% v/v) and 0.1 mL of the extract sample were mixed together in a test tube, to the mixture 0.05 mL of $FeSO_4$ solution was added to induce oxidative stress.

The mixture was incubated at 37ºC for 30 mins in an oven. 3 mL of TBA-TCA reagent was added and centrifuged at 3000 rpm for 15 mins. A pinkish-red chromogen formed and absorbance readings were recorded at 532 nm with the percentage inhibition then calculated using the equation below. (Ruberto, Baratta , Deans & Dorman, 2000)

$$\% \text{ Inhibition} = \frac{A(blank) - A(sample)}{A(blank)}\ x\ 100$$

Where:

$A_{(blank)}$ = absorbance of oxidised egg yolk without extract.

$A_{(sample)}$ = Absorbance of oxidised egg yolk with extract.

4.10 Statistical Analysis

GraphPad Instat was used for all data analysis. One-way Anova followed by Tukey-Kramer multiple comparison test, was used to determine difference in effects of extracts. All the results are presented as Mean ± SEM and considered to be statistically significant at $p < 0.05$.

4.11 Results and discussion

Solvent extraction

Upon sequential solvent extraction of the roots, different masses of crude extracts were obtained with EA extract (0.348 g) yielding the smallest mass and the MeOH (47.941 g) extract yielding the largest mass. The masses followed this order in terms of masses: EA < Hex < DCM < EtOH < MeOH (Table 4.1).

Table 4.1: Showing results of solvent extraction of the roots.

Extract	Physical appearance	Mass of extract (grams)	Percentage yield % (w/w)
n-Hexane	Yellowish and oily	0.668	0.267
Dichloromethane	Green and oily	0.803	0.321
Ethyl Acetate	Dark brown and solid	0.348	0.139
Methanol	Red and solid	35.091	14.026
70% Ethanol	Red and solid	47.941	19.162

Total phenolic content

The total phenolic content of the extracts was performed using the Folin Coicalteau reagent. The DCM extract (122.827 ± 0.010 µgGAE/mL) exhibited the largest total phenolic content while the MeOH extract (48.788 ± 0.001 µgGAE/mL) exhibited the least total phenolic content.

The order of content mass is as follows: MeOH < EtOH < Hex < EA < DCM and all extracts bar the hexane extract were statistically significant i.e. $p < 0.05$ (Figure 4.2). Extracts differed significantly ($p < 0.001$) in total phenolic content.

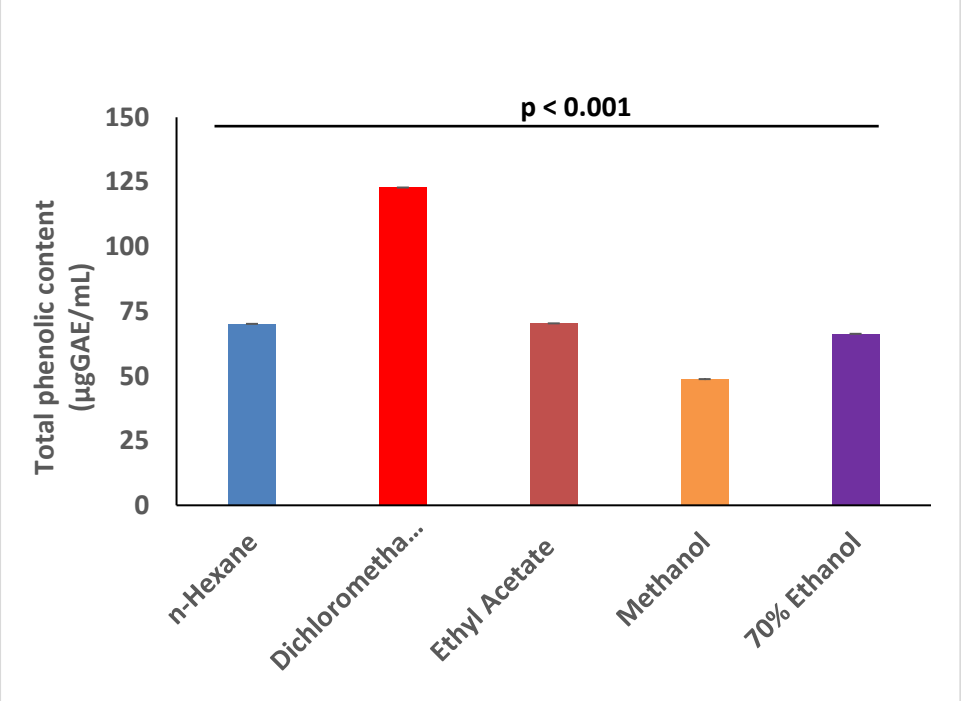


Figure 4.2: Total phenolic content of extracts of *P.prunelloides*.

Total Flavonoid Content

The principle behind this assay involves an aluminium chloride colorimetric reaction in which aluminium chloride forms acid stable complexes. The total flavonoid content of extracts was expressed as microgram quercetin equivalents per milligram of extract (µgQE/mg extract).

The DCM extract (88.543 ± 0.005 µgQE/mg exhibited) the largest content while the EtOH extract (19.254 ± 0.001 µgQE/mg of extract) exhibited the least content. The order of content mass is as follows: MeOH < EA < EtOH < Hex < DCM. The flavonoid content of extracts was statistically significant different in all extracts. (Figure 4.3).

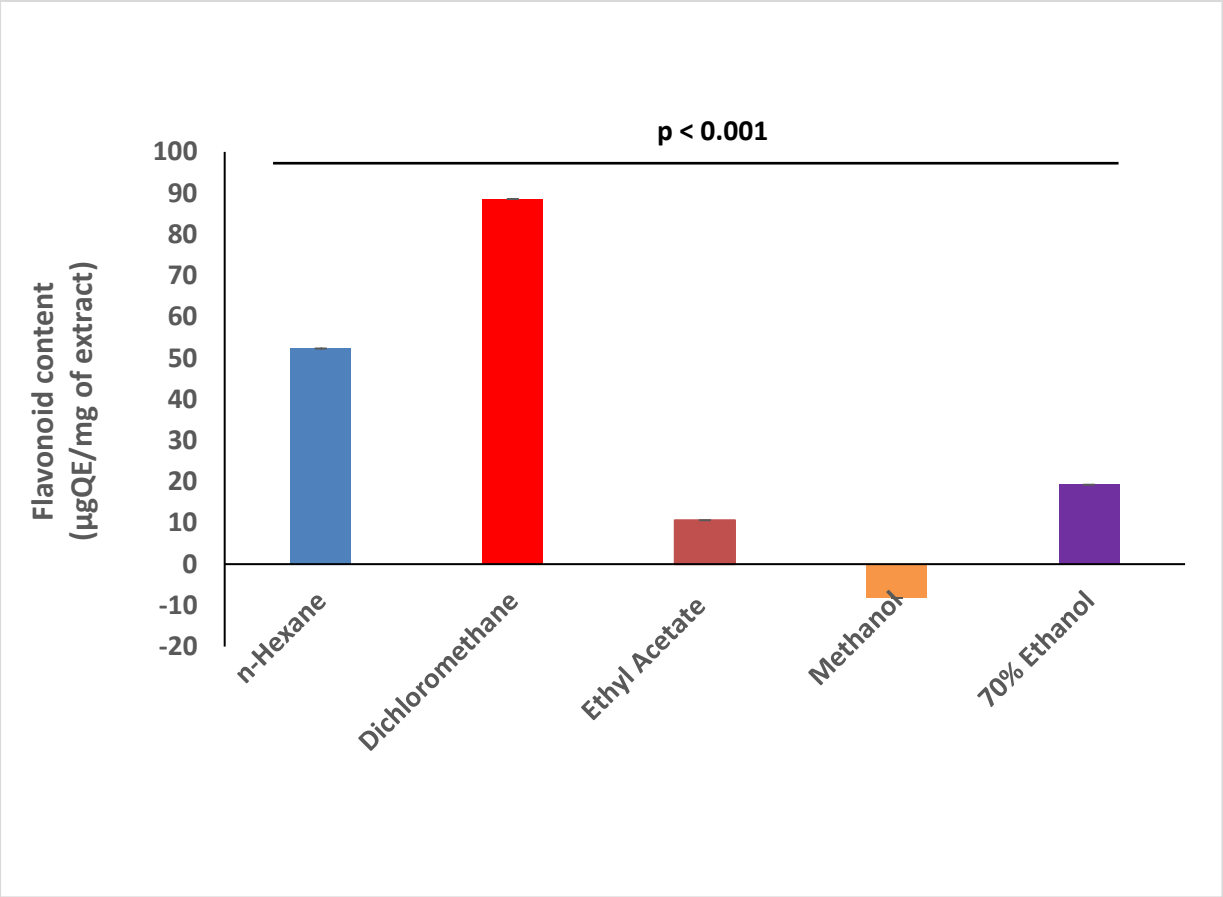


Figure 4.3: Flavonoid content of extracts of *P.prunelloides*

FRAP assay

The principle of this method is based on the reduction of a ferric tripyridyltriazine complex (Fe(III)-TPTZ) to its ferrous tripyridyltriazine complex form in the presence of anti-oxidants and the change in the colour intensity indicated antioxidant capacity.

The DCM extract (13.021 ± 0.008 AAE/mL) displayed the least FRAP ability while the MeOH extract (217.758 ± 0.025 AAE/mL) the highest. The order of content is as follows: DCM < Hex < EtOH < EA < MeOH and all extracts were statistically significant i.e. $p < 0.05$ (Figure 4.4).

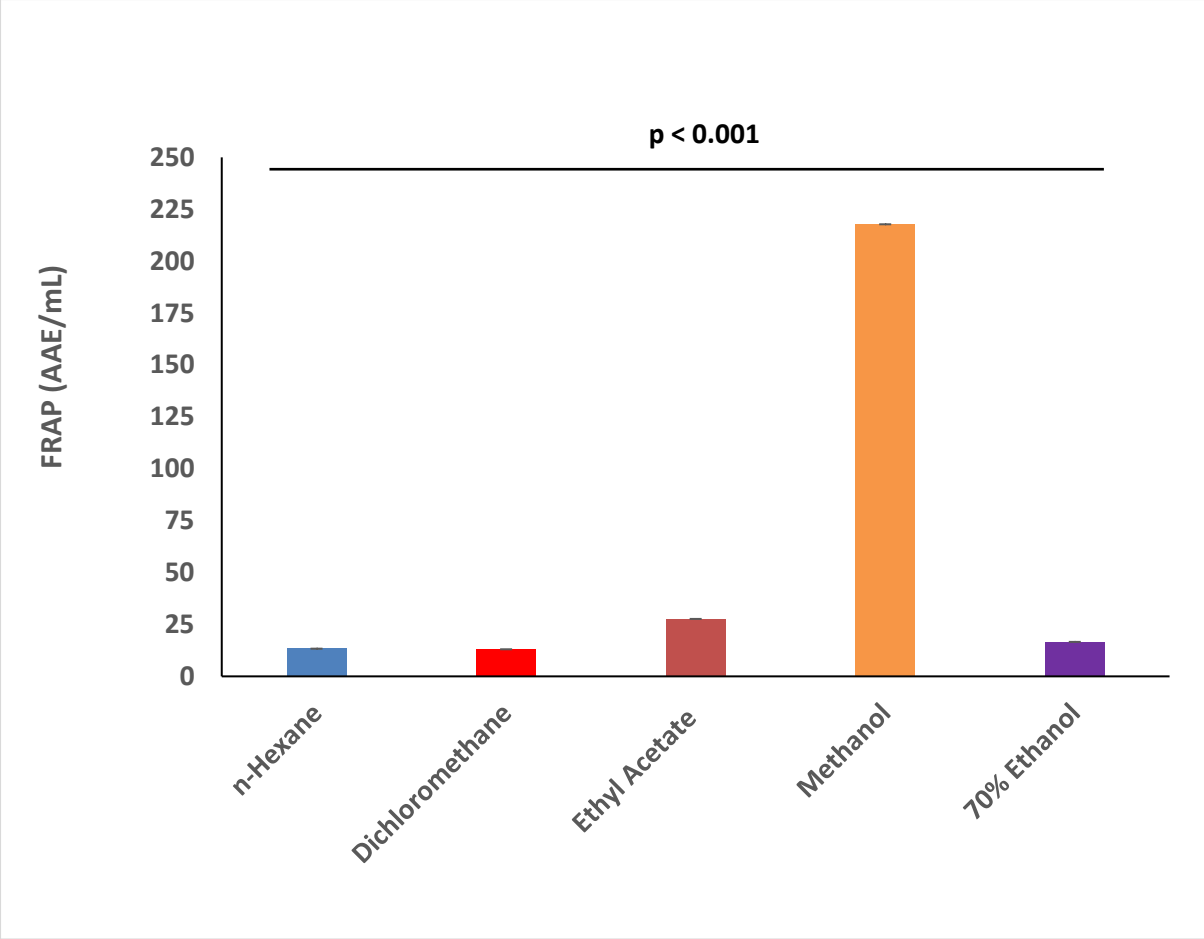


Figure 4.4 Total antioxidant capacity as measured by ferric reducing antioxidant power (FRAP) assay of *P.prunelloides*.

DPPH assay

The assay is based on the presumption that anti-oxidants react with 2,2-diphenyl-1-picrylhydrazyl (DPPH), it is a stable F.R., thus is reduced to the DPPHH and as a consequence of the reaction an absorbance at 517 nm that can be measured.

Table 4.2: Showing the % Inhibition and IC_{50} values of the extracts.

Concentration (mg/mL)	**n-Hex % Inhibition**	**DCM % Inhibition**	**EA % Inhibition**	**MeOH % Inhibition**	**EtOH % Inhibition**
0.03	1.292	8.118	5.812	61.439	2.583
0.06	1.199	3.137	7.934	63.469	3.506
0.125	1.199	4.705	20.572	82.380	7.380
0.25	0.461	5.996	26.292	87.362	14.852
0.5	1.91	12.270	41.421	87.362	23.155
1	0.0923	22.232	71.218	87.454	40.867
IC_{50} value (mg/mL)	0 ± 0.000	4244 ± 0.04	0.57 ± 0.021	0.006 ± 0.081	4.76±0.011

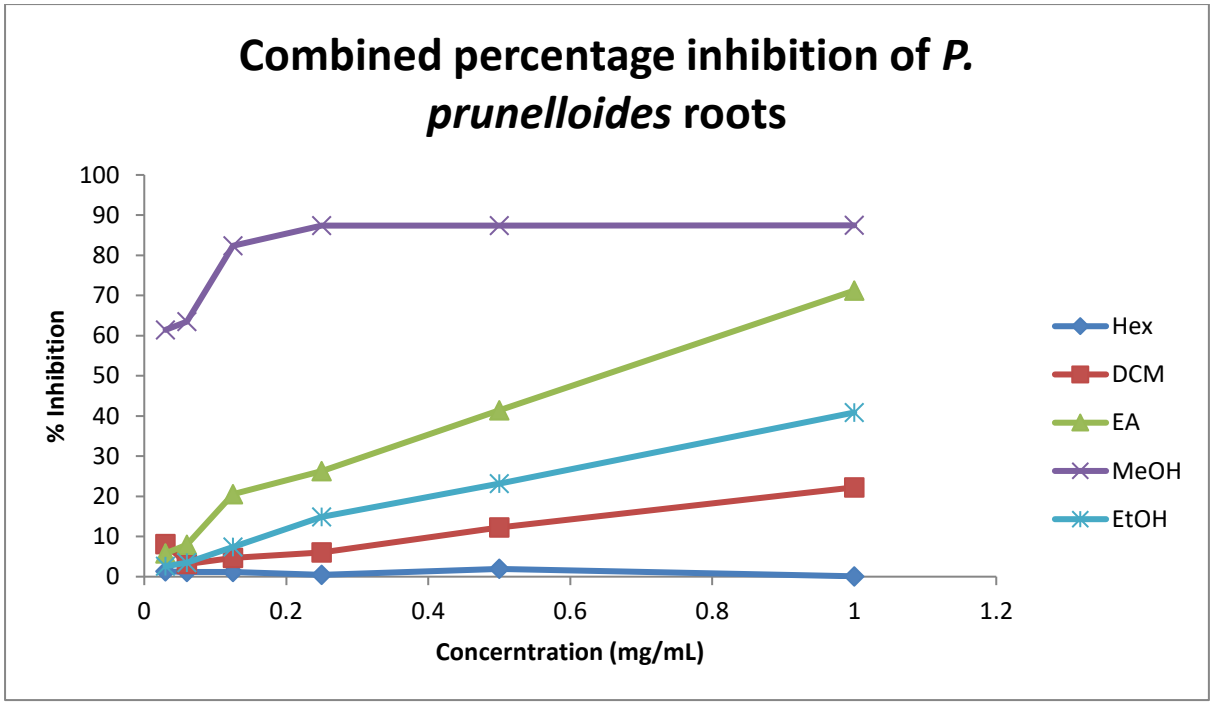
The percentage inhibition of the n-Hex extract ranged between 0.09230% and 1.931%, with an IC_{50} value of 0 ± 0.000 mg/mL showing an inability to scavenge the DPPH radical and also was statistically not significant (Table 4.2).

The percentage inhibition of the DCM extract ranged between 3.137% and 22.323%, with an IC_{50} value of 4244.081692 ± 0.044 mg/mL showing an inability to scavenge the DPPH radical and also was statistically not significant (Table 4.2).

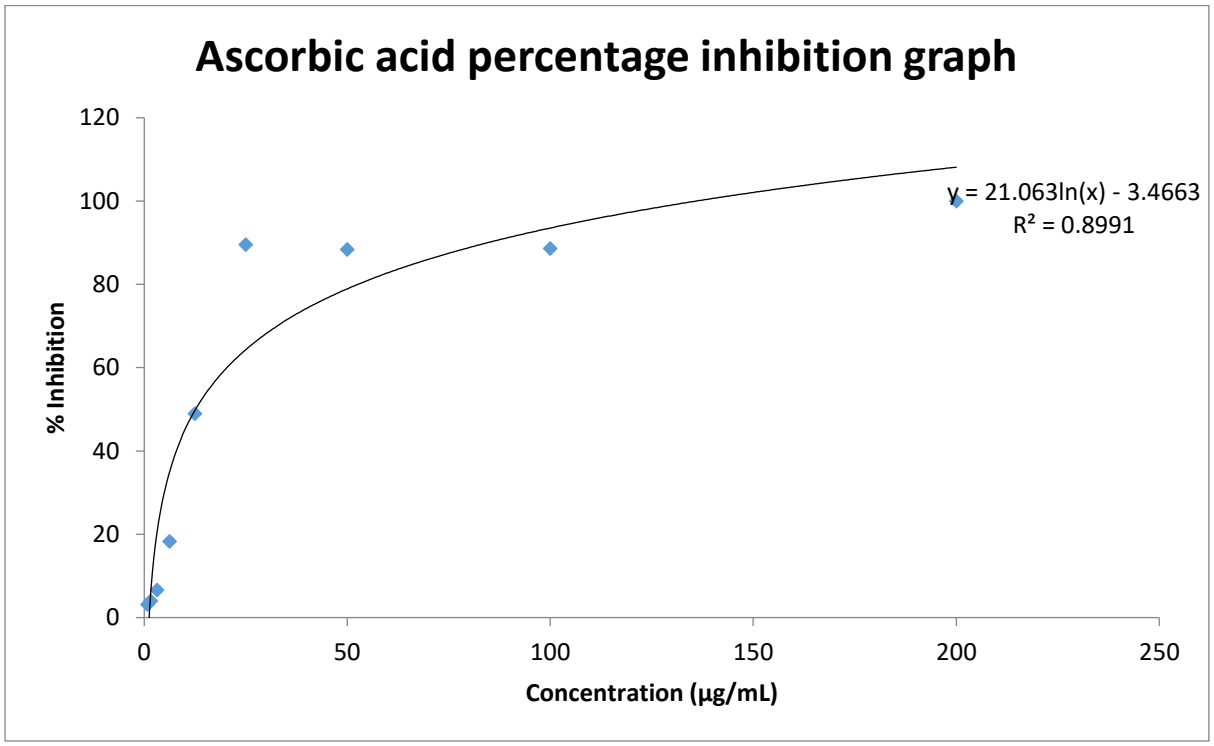
The percentage inhibition of the EA extract ranged between 5.812% and 71.218%, with an IC_{50} value of 0.579 ± 0.021 mg/mL showing an ability to scavenge the DPPH radical and also was statistically not significant (Table 4.2).

The percentage inhibition of the MeOH extract ranged between 61.439% and 87.454% showing an ability to scavenge the DPPH radical, with an IC_{50} value of 0.006 ± 0.081 mg/mL and also was statistically not significant (Table 4.2).

The percentage inhibition of the EtOH extract ranged between 2.583% and 40.867% showing an ability to scavenge the DPPH radical, with an IC_{50} value of 4.765 ± 0.011 mg/mL and also was statistically not significant (Table 4.2).



Graph 3: Showing % Inhibition of P. prunelloides extracts in DPPH assay



Graph 4: Showing standard curve % Inhibition of stock Ascorbic acid in DPPH assay

Lipid peroxidation (150 µg/mL)

All the extract were statistically significant with three inhibiting (i.e. n-Hex, DCM and EA) the peroxidation of lipids with the Hex extract (-36.395 ± 0.015%) showing the highest inhibition percentage while the DCM extract (-17.647 ± 0.005%) while the MeOH and EtOH extracts showed no inhibition.

The order of percentage inhibition is as follows: MeOH < EtOH < DCM < EA < Hex (Table 4.3)

Table 4.3: Showing results of lipid peroxidation (150 µg/mL) inhibition.

Extract	% Inhibition	P-value	Significance
n-Hexane	-36.395 ± 0.015	$4.998x10^{-4}$	Significant***
Dichloromethane	-17.647 ± 0.005	$4.694x10^{-2}$	Significant*
Ethyl Acetate	-26.190 ± 0.010	$1.866\ x10^{-2}$	Significant*
Methanol	26.871 ± 0.039	$3.434\ x10^{-2}$	Significant*
70% Ethanol	0.850 ± 0.019	$2.180\ x10^{-2}$	Significant*

*: p<0.05, **: p<0.01, ***: p<0.001

Lipid Peroxidation (300 µg/mL)

Only the EA (-12.881 ± 0.018%) and DCM (-0.847 ± 0.009%) extracts displayed inhibition but the rest did not, of the two extracts only the EA extract was statistically significant and the rest weren't statistically significant.

The order of percentage inhibition is as follows: EtOH < MeOH < Hex < DCM < EA (Table 4.4)

Table 4.4: Showing results of lipid peroxidation (300 µg/mL) inhibition.

Extract	% Inhibition	P-value	Significance
n-Hexane	5.424 ± 0.011	$9.553x10^{-1}$	Not significant
Dichloromethane	-0.847 ± 0.009	$5.522x10^{-1}$	Not significant
Ethyl Acetate	-12.881 ± 0.018	$1.526x10^{-2}$	Significant*
Methanol	6.610 ± 0.032	$1.857x10^{-1}$	Not significant
70% Ethanol	12.034 ± 0.024	$6.254x10^{-1}$	Not significant

*: $p < 0.05$

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Chapter 5

5.1 Conclusions

The extract of *H. pauciflorus* was very fatty and rendered the isolation of compounds very difficult within the time frame available for the project and the equipment available for isolation. Column chromatography of the hexane extract did not result in the isolation of compounds TLC showed that possible isolates.

The presence of flavonoids and phenolic compounds was quantified by means of a flavones and flavonols assay and Folin-Ciocalteau assay respectively. The study has indicated the presence of both compounds in both plant samples.

According to the FRAP assay, both samples were able to act as anti-oxidants or as reductants whilst they could not significantly act as anti-oxidants or reductants according to the DPPH assay though both plant samples do show an ability to scavenge free radicals on a dose-dependent manner.

More work can be done to significantly establish the ability of both samples to scavenge the DPPH radical.

At the concentration of 150 µg/mL is where both samples were able to inhibit lipid peroxidation best when compared to their ability to inhibit lipid peroxidation at 300 µg/mL.

This ability to inhibit lipid peroxidation may account for their capability to reduce Fe^{3+} to Fe^{2+} and why they are able to act as lipid peroxidation inhibitors.

The use of vital parts of plants such as roots, bark, tubers, etc. has to be halted but most likely should be decreased to ensure plant species survival. More should be done to classify them according to IUCN guidelines to aid with the sustainable utilisation plant species more so wild plant species.

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